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Intracellular Signaling Through the Membrane-Type I Matrix  
Metalloproteinase

PRINCIPAL INVESTIGATOR: Paolo Mignatti, Ph.D.

CONTRACTING ORGANIZATION: New York University School of Medicine  
New York, New York 10016

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## INTRODUCTION.

The membrane-type 1 matrix metalloproteinase (MT-MMPs), a member of the MMP family of proteinases, has been implicated in the invasiveness and metastatic potential of a variety of human malignancies, including mammary carcinoma [1-4]. Unlike the other members of the matrix metalloproteinase (MMP) family, which are secretory proteins, MT1-MMP is a transmembrane proteinase with the catalytic domain exposed on the cell surface and a short (20–26 amino acid) cytoplasmic domain [5]. On the tumor cell surface MT1-MMP forms a tri-molecular complex with MMP-2 and its physiological inhibitor, the tissue inhibitor of metalloproteinases-2 (TIMP-2) [6]. Thus, MT1-MMP acts as a membrane binding site for MMP-2•TIMP-2 complex. Binding of the enzyme-inhibitor complex to MT1-MMP is required for MMP-2 activation [6]. MMP-2 interaction with MT1-MMP has analogy to the high-affinity binding of urokinase plasminogen activator (uPA) to its cell membrane receptor (uPAR) [7]. uPA and uPAR have also been implicated in the invasion and metastasis of a variety of tumors including breast carcinoma [8-11]. Although uPAR is not a transmembrane protein [12], binding of uPA generates intracellular signals that control cell functions including migration and proliferation through mechanisms independent of the proteolytic activity of uPA [13, 14]. Other transmembrane proteins with short cytoplasmic domains similar to MT1-MMP – the integrins – also generate intracellular signals following interaction with their extracellular ligands [15, 16]. Based on these analogies, this project aims to test the hypothesis that MT1-MMP binding of MMP-2 and/or TIMP-2 generates intracellular signaling through interaction of the cytoplasmic domain of MT1-MMP with signaling proteins, and thus regulates cell functions involved in breast carcinoma progression.

## BODY

To study the effect of MMP-2 and TIMP-2 binding to MT1-MMP on intracellular signaling and the control of cell migration and proliferation, we sought to obtain a panel of cells with differing levels of MT1-MMP. Our original strategy described in Task 1 of the Statement of Work involved the selection of clones of MDA-MB-231 or MDA-MB-435 cells. Because this procedure turned out to be labor intensive and time consuming, we adopted two alternative strategies: 1) the use of a panel of human HT1080 fibrosarcoma cells stably transfected with sense or antisense MT1-MMP cDNA, which had previously been generated and characterized in our laboratory; and 2) the generation of an inducible system, in which MT1-MMP expression can be modulated in MCF-7 human mammary carcinoma cells by varying the concentration of Doxycycline, a tetracycline analog, in the culture medium.

A panel of MT1-MMP transfectant clones was generated in our laboratory by transfection with expression plasmids containing nucleotides 1-2369 of MT1-MMP cDNA (pc3SE) or the corresponding antisense cDNA (pc3AS) under transcriptional control by the CMV promoter in the pcDNA3 vector [17] (obtained through our collaboration with Dr. Jorma Keski-Oja, University of Helsinki Department of Pathology, Finland). Non-transfected cells or control cells transfected with the vector alone constitutively express 60 kDa and 58 kDa MT1-MMP. The levels of these MT1-MMP forms are dramatically reduced in clones of cells transfected with the antisense cDNA.

Conversely, clones of cells transfected with the sense cDNA express high levels of 60 kDa and 58 kDa MT1-MMP, in addition to the 63 kDa proenzyme and a 43 kDa degradation product [18]. Sense and antisense MT1-MMP transfectant clones also differ in their expression of active MMP-2. Non-transfected HT1080 cells or control cells transfected with the vector alone and antisense transfectants secrete MMP-2 only in its inactive, 72 kDa form. In contrast, in the conditioned medium of cells transfected with MT1-MMP cDNA 68/66 kDa intermediate activation forms of MMP-2 can be detected in addition to the 72 kDa proenzyme (Fig. 1). Control, vector-transfected cells and MT1-MMP transfectants have cell-associated MMP-2, as assessed by gelatin zymography of cell extracts. In contrast, antisense transfectants have virtually no cell-associated MMP-2, although they secrete amounts of MMP-2 comparable to those of the other cell clones (Fig. 2).

Because TIMP-2 is an extracellular ligand for MT1-MMP, and TIMP-2 and  $\alpha_v\beta_3$  integrin have been implicated in the cell surface binding and activation of proMMP-2 [6, 19, 20], we characterized our panel of MT1-MMP transfectant cell clones for the expression of these proteins (Fig. 3). By Western blotting control cells and antisense MT1-MMP cDNA transfectants secreted comparable amounts of TIMP-2. In contrast, the levels of TIMP-2 secreted by MT1-MMP-transfected cells were dramatically lower (Fig. 3, lanes 5-7). However, control and antisense or sense transfectants contained comparable amounts of cell-associated TIMP-2, showing that the low TIMP-2 levels in the conditioned medium of MT1-MMP transfectants did not result from increased TIMP-2 binding to MT1-MMP or the extracellular matrix. Similar differences were observed in the amounts of secreted TIMP-1, which was undetectable in the conditioned medium of MT1-MMP transfectants (see Fig 7 F). Western blotting analysis of  $\alpha_v\beta_3$  integrin expression showed that the levels of the  $\alpha_v$  and  $\beta_3$  integrin chains were considerably lower in the MT1-MMP transfectants than in the other cell clones (Fig. 3).

To investigate whether the downregulation of  $\alpha_v\beta_3$  and TIMP-2 resulted from increased MMP activity in the MT1-MMP transfectants, we analyzed the expression of these proteins in cells grown in the presence of the MMP inhibitor Marimastat (10  $\mu$ M). In the presence of Marimastat all the clones contained comparable levels of secreted TIMP-2, implicating MMP activity in TIMP-2 downregulation. In contrast, Marimastat had no effect on  $\alpha_v\beta_3$  levels, indicating that  $\alpha_v$  and  $\beta_3$  gene expression may be downregulated in our MT1-MMP transfectants (Fig. 4).

Our previous work has shown that plasmin activates proMMP-2 in the presence but not in the absence of cells, suggesting a role for the cell surface in this process [21]. To investigate the potential role of MT1-MMP in plasmin-mediated activation of proMMP-2, the transfected cell clones were incubated in the presence or absence of plasminogen (4  $\mu$ g/ml) for 16 h. HT1080 cells secrete high levels of uPA that rapidly convert plasminogen into plasmin [21; data not shown]. Gelatinase activation was analyzed both by degradation of a specific fluorogenic substrate and by gelatin zymography.

Preliminary experiments showed that the fluorogenic assay was linear with volumes of conditioned medium ranging 10-100  $\mu$ l and incubation times ranging 0.5 – 3.0 h (Fig. 5). Addition of EDTA to the reaction mixture or depletion of the gelatinases from the conditioned medium by gelatin-Sepharose chromatography blocked substrate

degradation (Fig. 6), showing the specificity of the assay for the gelatinases. Consistent with previous findings [21], medium conditioned by non-transfected HT1080 cells in the presence of plasmin(ogen) showed a 4.5-fold increase in gelatinase (MMP-2 and MMP-9) activity relative to medium conditioned in the absence of plasminogen (Fig. 5).

With control, vector-transfected cells the activity measured in the presence of plasmin(ogen) was similar to that of MT1-MMP cDNA-transfected cells in the absence of plasmin(ogen) (Fig. 6). With MT1-MMP transfectants addition of plasmin(ogen) increased the gelatinase activity considerably. In contrast, with antisense cDNA transfectants plasmin had a minor effect on the gelatinase activity. With all the three clones, the activity was completely abolished by EDTA or by pretreatment of the conditioned media with gelatin-Sepharose, showing that the activity measured by this assay in medium conditioned by the different transfectants was mediated exclusively by the gelatinases.

By gelatin zymography medium conditioned in the absence of plasmin(ogen) by MT1-MMP transfectants (Fig. 7, MT1-MMP SE) showed three MMP-2 bands of 72, 68/66 and 64/62 kDa, whereas control, vector-transfected cells and antisense cDNA transfectants only showed the 72 kDa band (Fig. 7, V and MT1-MMP AS). Addition of plasmin(ogen) to the culture medium of antisense transfectants had no effect on proMMP-2 activation, although it generated active (84/82 kDa) MMP-9 (Fig. 7 C). In contrast, plasmin(ogen) added to the culture medium of control or MT1-MMP-transfected cells generated active (64/62 kDa) MMP-2 (Fig. 7 A, B and D). ProMMP-2 activation by plasmin was much more efficient with MT1-MMP transfectants than with control, vector-transfected cells (Fig. 7 D). Thus, plasmin activates MMP-2 in cells that express MT1-MMP but has no effect on the proMMP-2 of cells with very low levels (or virtually devoid) of MT1-MMP.

In the presence of plasmin(ogen) antisense cDNA transfectants showed no active MMP-2 but secreted active (84/82 kDa) forms of MMP-9 (Fig. 7 C). This finding indicates that the low level of gelatinase activity measured by the fluorogenic substrate assay in medium conditioned by these cells was mediated only by MMP-9. MT1-MMP transfectants had a level of MMP-9 much lower than the other two clones. In the presence of plasmin(ogen) MT1-MMP transfectants did not show MMP-9 activation (Fig. 7), an unexpected but reproducible effect. Thus, the activity measured by the fluorogenic assay in the conditioned medium of these cells was mediated only or predominantly by MMP-2.

These findings suggested that the effect of plasmin on MMP-2 activation could be mediated either indirectly by cleavage/activation of cell surface proteins such as MT1-MMP,  $\alpha_v\beta_3$  or TIMP-2, or directly by activation of cell surface-associated proMMP-2. Plasmin had no effect on the levels of  $\alpha_v$  or  $\beta_3$  integrin chains expressed by all the transfectant cell lines (Fig. 8 A and B) but dramatically downregulated the amount of TIMP-2 in the conditioned medium of the MT1-MMP transfectants, which secrete a low amount of this inhibitor (see Fig. 3 A). However, plasmin did not appear to significantly reduce the levels of TIMP-2 in the other two clones, which expressed considerably higher amounts of the inhibitor (Fig. 8 C). Thus, plasmin activation of proMMP-2 does not appear to be mediated by proMT1-MMP activation or by cleavage or degradation of  $\alpha_v\beta_3$  integrin or TIMP-2.



To characterize the relative contribution of plasmin and MT1-MMP to proMMP-2 activation we tested the effect of several proteinase inhibitors. Addition to MT1-MMP transfectants of 1,10-phenanthroline (10  $\mu$ g/ml) or Marimastat (1  $\mu$ M), which inhibit metalloproteinases, resulted in decreased levels of partially active (68/66 kDa) MMP-2 (Fig. 7 B), consistent with previous findings that proMMP-2 processing into its 68/66 kDa form is MT1-MMP-dependent and that conversion to fully active, 62 kDa MMP-2 occurs through autocatalysis [6]. Addition of plasmin(ogen) in the presence or absence of the metalloproteinase inhibitors resulted in the conversion of partially active (68/66 kDa) MMP-2 to fully active (64/62 kDa) MMP-2. This result indicates that plasmin can catalyze the generation of active, 62 kDa MMP-2 under conditions in which MT1-MMP activity and/or autocatalysis are inhibited. Conversely, the effect of plasmin(ogen) was inhibited by addition of aprotinin (100  $\mu$ g/ml), a serine proteinase inhibitor that blocks plasmin activity (Fig. 7 A and B). These results showed that plasmin directly catalyzes the generation of 64/62 kDa MMP-2 from its intermediate activation form (68/66 kDa) generated by MT1-MMP and from the 72 kDa proenzyme.

*The results discussed above have been submitted for publication in Biochemical Journal. A copy of the manuscript is enclosed in the Appendices section of this report.*

All our MT1-MMP transfectants, which expressed high levels of MT1-MMP, had a very low expression of  $\alpha_v\beta_3$  integrin, an effect independent of the high proteolytic activity of these cells. This observation suggests that the downregulation of this integrin does not reflect clonal variability but may result from decreased gene expression, and that this effect may be mediated by signaling through MT1-MMP. To test this hypothesis we will use the Tet-off inducible system described below.

To test our hypothesis that binding of extracellular ligands – MMP-2 and/or TIMP-2 – to MT1-MMP can modulate cell functions through proteolysis-independent mechanisms we characterized our MT1-MMP transfectants for cell proliferation and migration. To abolish differences in the proteolytic activity of the different clones, proliferation and migration assays were done in the presence of 1  $\mu$ M marimastat.

MT1-MMP transfectant clones expressing high levels of MT1-MMP showed growth rates higher than control, vector transfected cells. In contrast, the proliferation rate of antisense MT1-MMP cDNA-transfected cells that express virtually no MT1-MMP was much lower than that of control cells (Fig. 9).

To test if differences in motility also exist among our transfectant clones, we characterized their ability to migrate spontaneously in modified blind well (Boyden chamber) assays with 5- $\mu$ m-pore polycarbonate filters. The results showed that transfectants expressing high levels of MT1-MMP have several-fold increased motility relative to control cells. In contrast, antisense transfectants are virtually devoid of migratory ability (Fig. 10).

As discussed above, all our MT1-MMP transfectants express differing levels of the MT1-MMP ligands, MMP-2 and TIMP-2. To test if binding of these ligands to MT1-MMP can generate intracellular signals, we added excess (50 nM) recombinant MMP-2 and TIMP-2 to control, vector-transfected cells and to sense or antisense MT1-MMP transfectants in the presence of marimastat (1  $\mu$ M). Because our initial observations

indicated a potential interaction with regulatory component(s) of the Ras signaling pathways, we analyzed the activation of the extracellular signal-regulated protein kinase-1 and -2 (ERK-1 and -2) by Western blotting with antibodies to human phosphorylated ERK (New England Biolabs) and with control polyclonal antibodies to total human ERK-2. Our recent, preliminary results showed that addition of MMP-2 and TIMP-2 to vector-transfected cells induced a significant activation of ERK-1 and -2 within 15-30 min of treatment, an effect that was maintained for 1 h before the level of activation went back to control level. However, addition of MMP-2 and TIMP-2 to cells transfected with antisense MT1-MMP cDNA had no effect on ERK activation. We are presently repeating these experiments to confirm these results. Although indirectly, this observation provides an indication that interaction of MT1-MMP with its extracellular ligands is followed by the activation of the ERK pathway of intracellular signaling. It is noteworthy that in a variety of cell types this signaling pathway controls proliferation and migration, cell functions that are upregulated in our MT1-MMP overexpressing cells, and downregulated in our antisense MT1-MMP transfectants.

Because the effects discussed above may reflect clonal variation, we are constructing an inducible expression system for modulating MT1-MMP expression in breast cancer cells. For this purpose, human MCF-7 breast carcinoma cells that stably express high levels of the tetracycline-controlled transactivator were purchased from Clontech. These cells express extremely low levels of MT1-MMP, as assessed by Western blotting. A 2369 bp MT1-MMP cDNA insert in pcDNA3 [17] (MTpc3SE, obtained through our collaboration with Dr. Jorma Keski-Oja, University of Helsinki Department of Pathology, Finland) was excised by EcoRI digestion followed by treatment with the Klenow enzyme. The excised cDNA was bluntly ligated in either the sense or antisense orientation in to the EcoRV site of the tetracycline-responsive expression vector pTRE2 (Clontech). The sense construct was cotransfected with the pTK-Hyg hygromycin resistance vector (Clontech) in MCF-7 cells using Eugene (Roche Molecular Biochemicals). Clones of G418- and hygromycin-resistant cells are being selected. Resistant clones will be subcloned in the absence and in the presence of 1  $\mu$ g/ml of doxycycline in order to identify the clones that have both the lowest expression of MT1-MMP in the presence and the highest expression of MT1-MMP in the absence of the antibiotic.



## KEY RESEARCH ACCOMPLISHMENTS

- Generation of a panel of tumor cells that express different levels of MT1-MMP.
- Construction of a vector that permits expression of MT1-MMP under control by the tetracycline resistance transactivator.
- Indications that high levels of MT1-MMP expression are associated with high levels of tumor cell proliferation and migration, whereas in cells expressing low levels of MT1-MMP proliferation and migration are reduced, relative to control cells.
- Demonstration that tumor cells with high expression of MT1-MMP have reduced levels of TIMP-2, the physiological inhibitor of MT1-MMP; high levels of MMP activity being responsible for TIMP-2 degradation.
- Indications that tumor cells with high expression of MT1-MMP have decreased expression of  $\alpha_v\beta_3$  integrin through a proteolysis-independent mechanism (that may involve decreased gene expression).
- Indications that binding of the extracellular ligands of MT1-MMP, MMP-2 and TIMP-2, results in rapid activation of the ERK pathway of intracellular signaling.
- Demonstration that MT1-MMP and plasmin act in concert to activate proMMP-2.
- Demonstration that plasmin activation of proMMP-2 requires the expression but not the catalytic activity of MT1-MMP.
- Demonstration that cell-associated proMMP-2 is activated by plasmin, whereas secreted proMMP-2 is not.
- Demonstration that proMMP-2 activation by plasmin does not result from plasmin-mediated MT1-MMP activation and is independent of  $\alpha_v\beta_3$  integrin or TIMP-2 levels.

## REPORTABLE OUTCOMES

Sara Monea, Kaisa Lehti, Jorma Keski-Oja and Paolo Mignatti, "Membrane-Type 1 Matrix Metalloproteinase Is Required for Plasmin-Mediated Activation of Gelatinase A", manuscript submitted for publication in Biochemical Journal (Nov. 2000).

## CONCLUSIONS

The results obtained have provided several indications that MT1-MMP may control a variety of tumor cell functions through proteolysis-independent mechanisms. These indications include: a) the observation that high levels of MT1-MMP expression are associated with high levels of tumor cell proliferation and migration, whereas in cells expressing low levels of MT1-MMP proliferation and migration are reduced, relative to control cells; b) the finding that tumor cells with high expression of MT1-MMP have decreased expression of  $\alpha_v\beta_3$  integrin through a proteolysis-independent mechanism (that may involve decreased gene expression); c) the preliminary observation that binding of the extracellular ligands of MT1-MMP, MMP-2 and TIMP-2, results in activation of the ERK pathway of intracellular signaling; d) the demonstration that plasmin activation of proMMP-2 requires the expression but not the catalytic activity of MT1-MMP.

These points require further investigation to confirm our data and understand the mechanism(s) through which MT1-MMP may transduce intracellular signals. For this purpose we are constructing a Tet-off system that will afford to modulate MT1-MMP levels in human MCF-7 mammary carcinoma cells. This experimental model will allow us to rule out the possibility that our observations reflect clonal variations, rather than being indicative of effects caused exclusively by different levels of MT1-MMP expression. In particular, this system will provide a useful tool to study the effect of addition of MMP-2/TIMP-2 on the activation of ERK in cells expressing differing levels of MT1-MMP.

Our observations on the relative contribution of plasmin and MT1-MMP to MMP-2 activation have shown that this process requires MMP-2 binding to, but not the catalytic activity of MT1-MMP. We also found that cells expressing high levels of MT1-MMP but virtually no  $\alpha_v\beta_3$  integrin, a cell surface binding site for MMP-2, bind and activate this gelatinase. These findings support the hypothesis that MT1-MMP acts as a cell membrane binding site for MMP-2•TIMP-2 complex. These two proteins may represent physiological ligands for MT1-MMP: as suggested by our findings, their binding may generate intracellular signals independent of the proteolytic activity of MT1-MMP and/or MMP-2. Other physiological ligands – for example extracellular matrix proteins – might also contribute to this process. We will consider investigating this hypothesis in our future experiments with the Tet-off system in MCF-7 cells.

Our project aims to test the hypothesis that a transmembrane proteinase, MT1-MMP, plays a role in tumor progression independent of its proteolytic activity. The results so far obtained suggest that this hypothesis may be correct. Our future work will therefore be aimed to elucidate the potential mechanisms of signal transduction initiated by MMP-2 and/or TIMP-2 binding to MT1-MMP. On the basis of these and future results, novel pharmacological tools can be designed which will be aimed at specifically inhibiting MMP-2 and/or TIMP-2 binding to MT1-MMP, or to block the intracellular signal transduction pathways triggered by the enzyme/inhibitor interaction with the cell membrane.

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## **APPENDICES**

Original halftones (Figures 1-4, 7,8) are attached with pages 59-66 of the original of this report.

FIG. 1

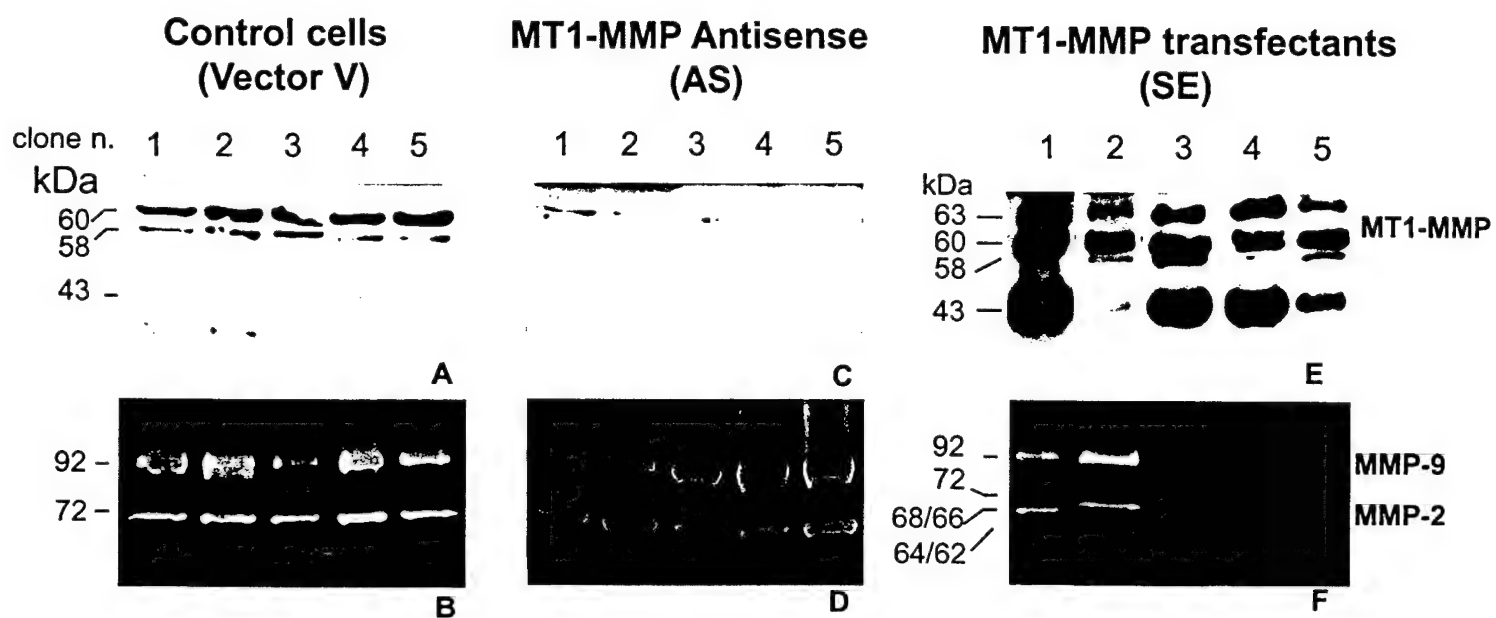
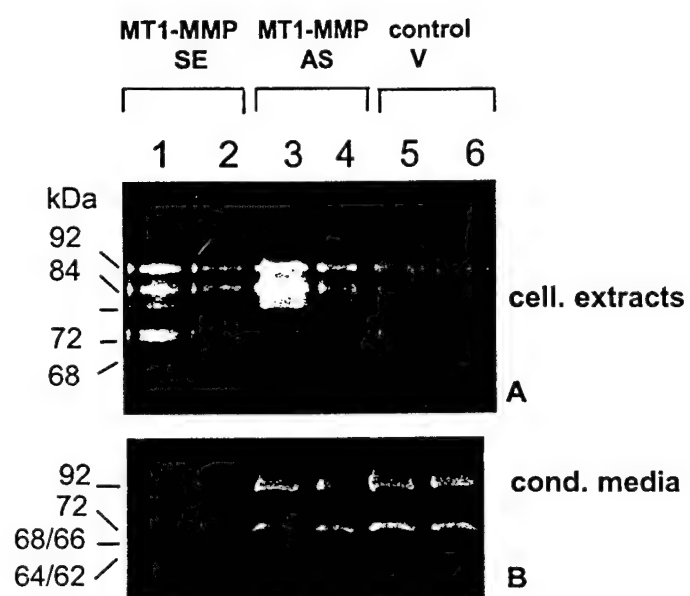
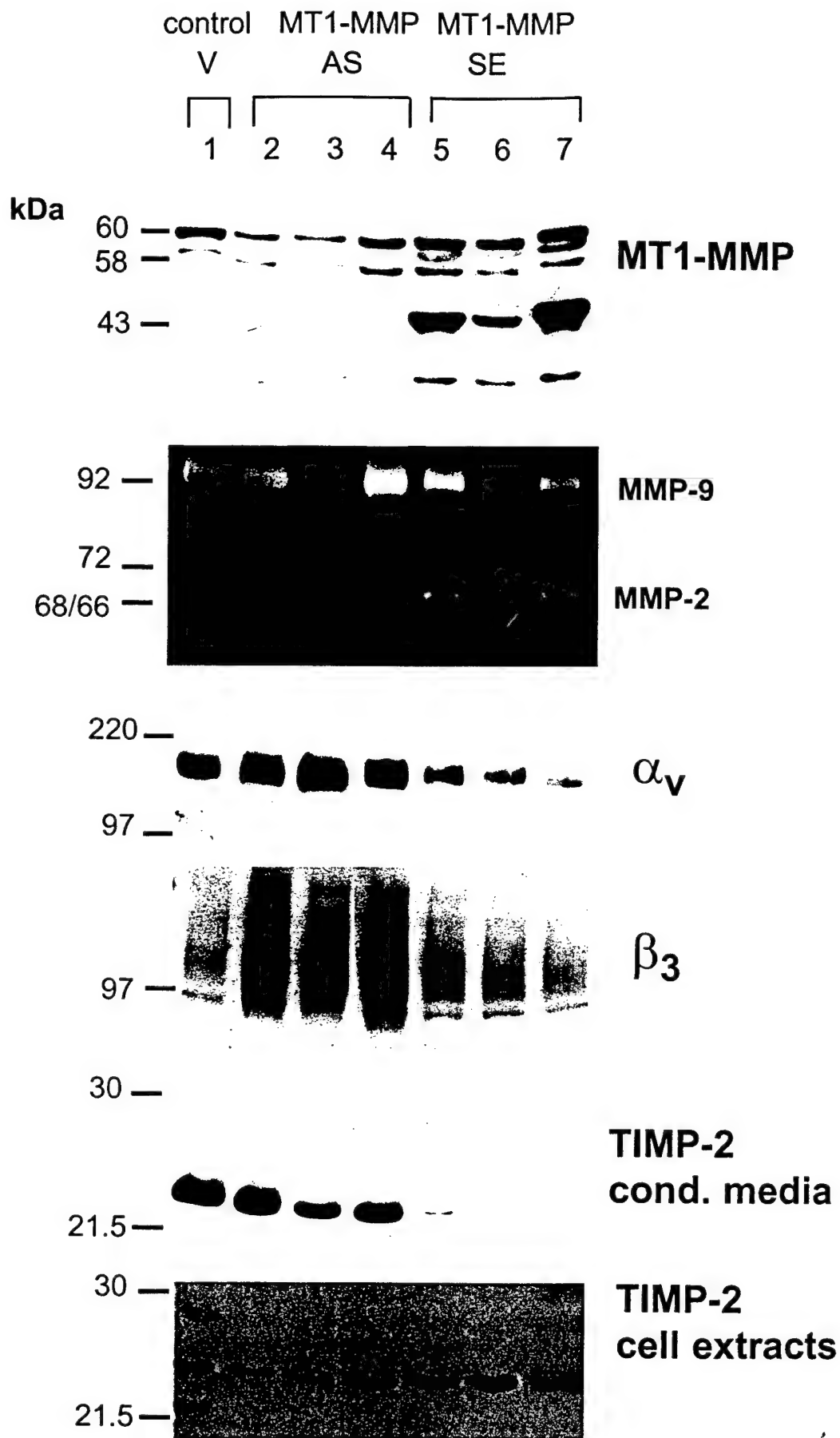


FIG. 2







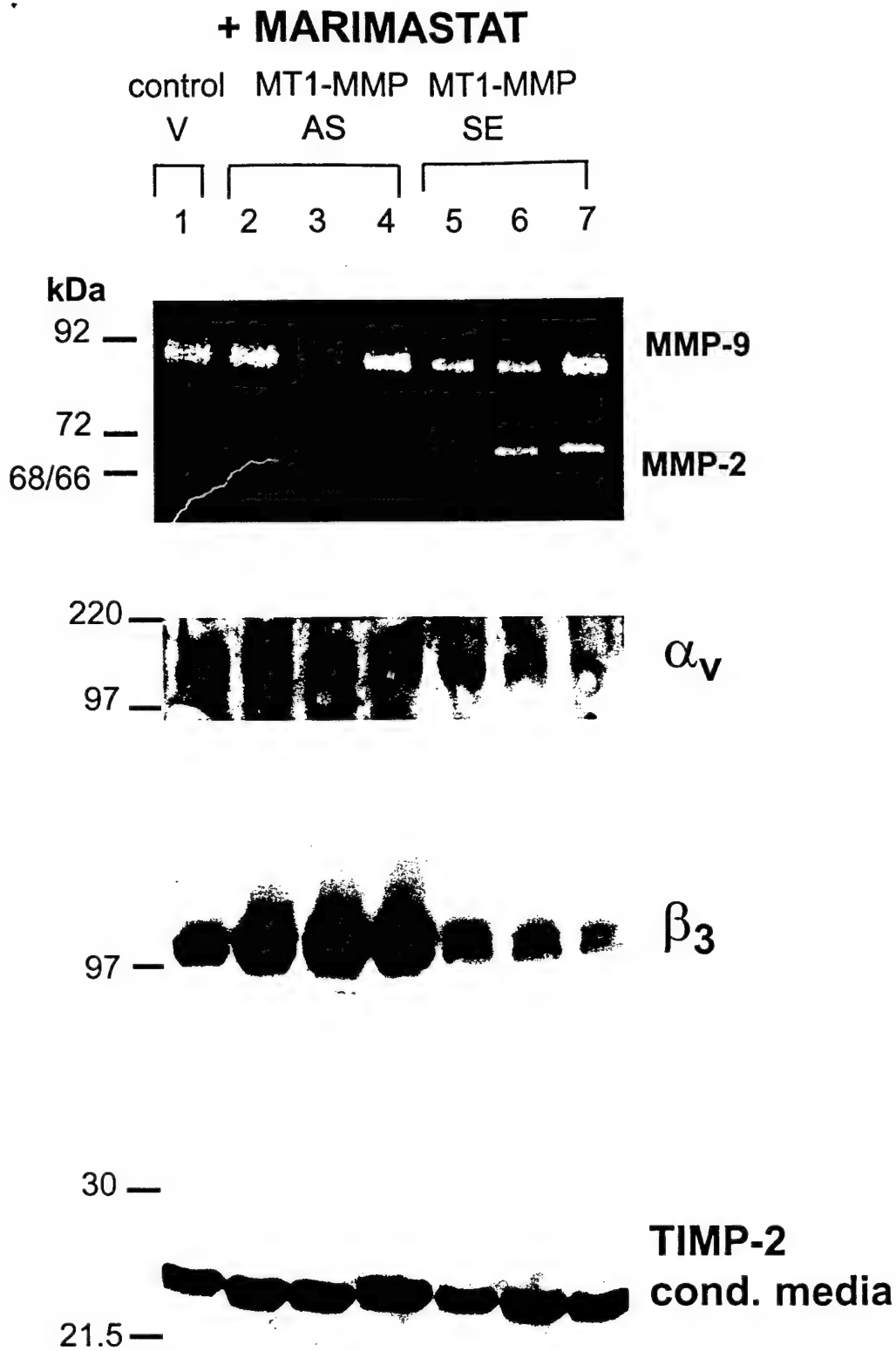


FIG. 5

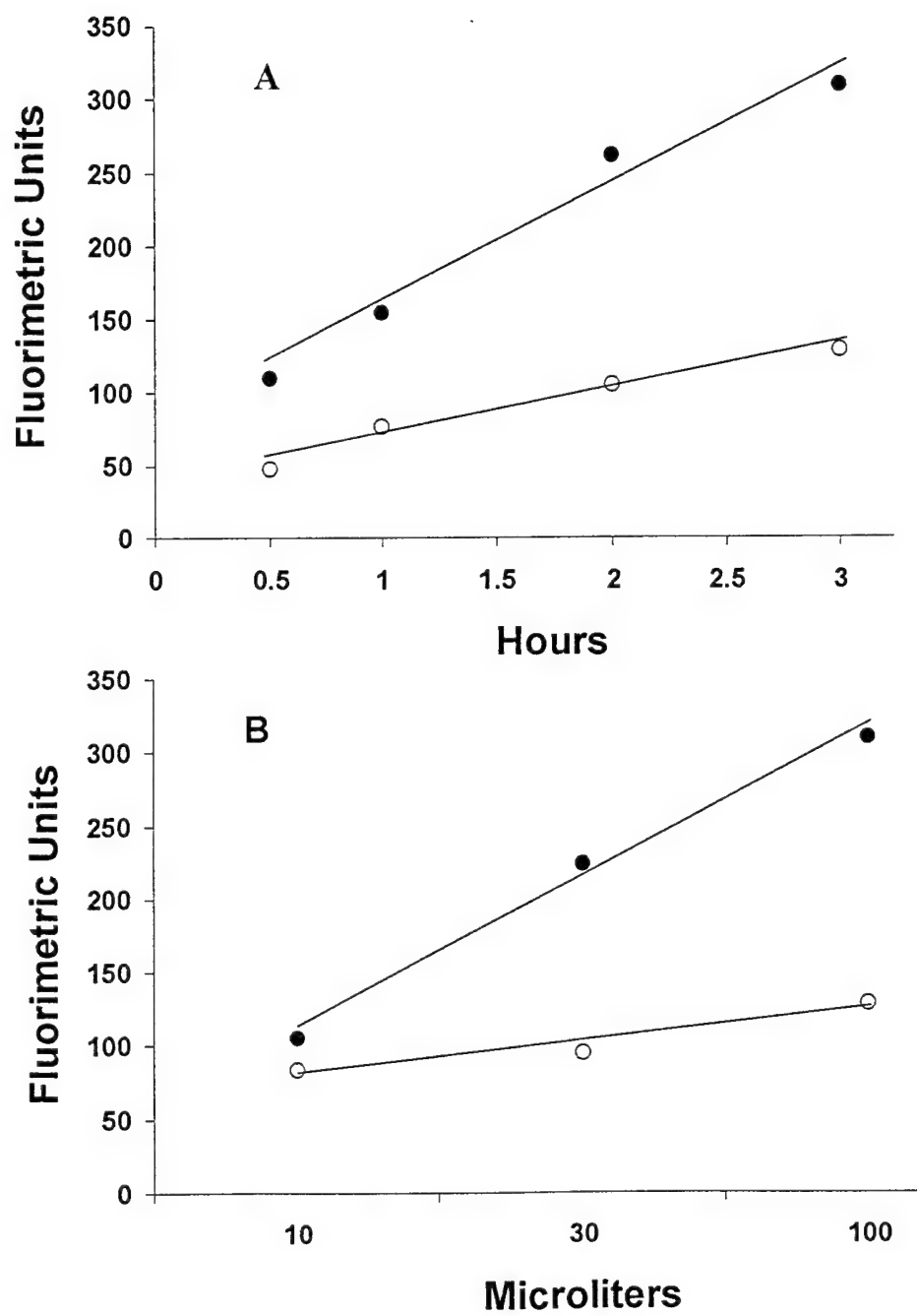
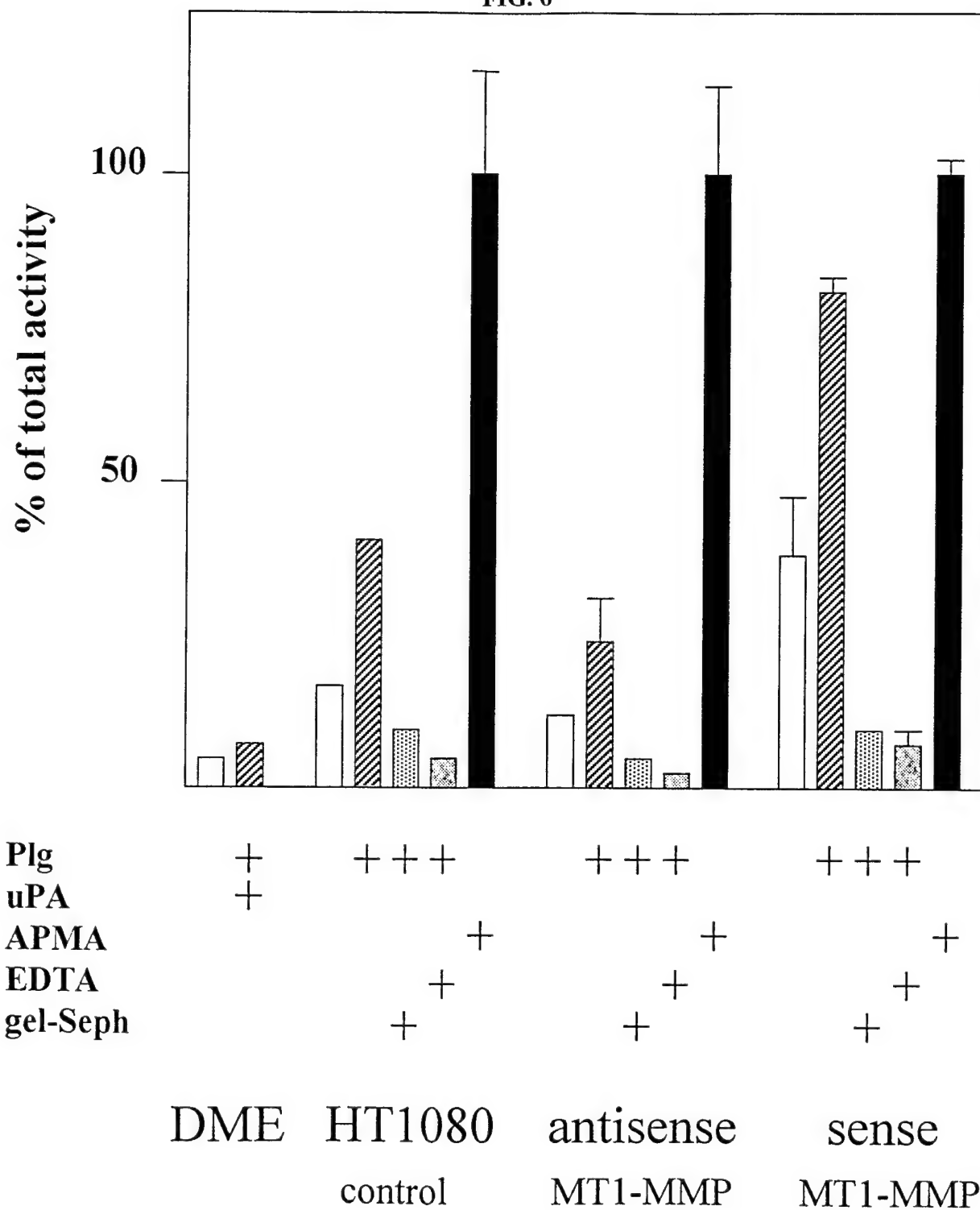


FIG. 6



Phenanthroline	-	+	+	-	-
Plasminogen	-	-	+	+	+
Aprotinin	-	-	-	-	+

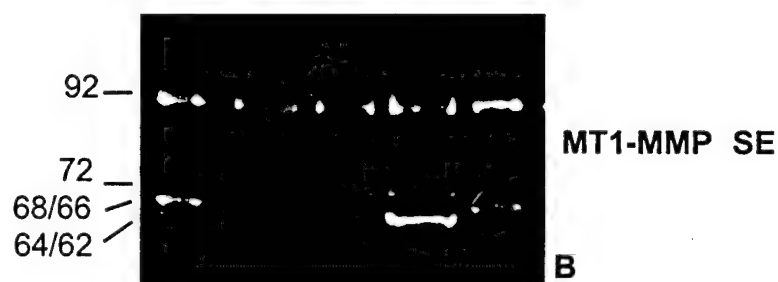
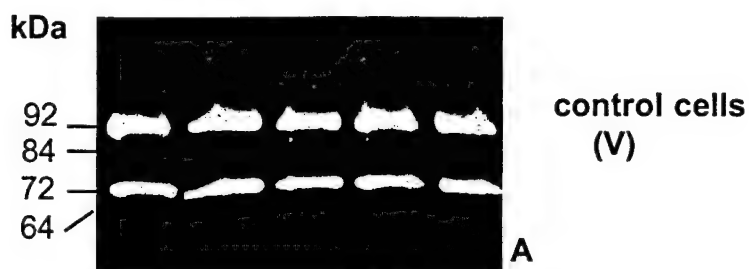


FIG. 8

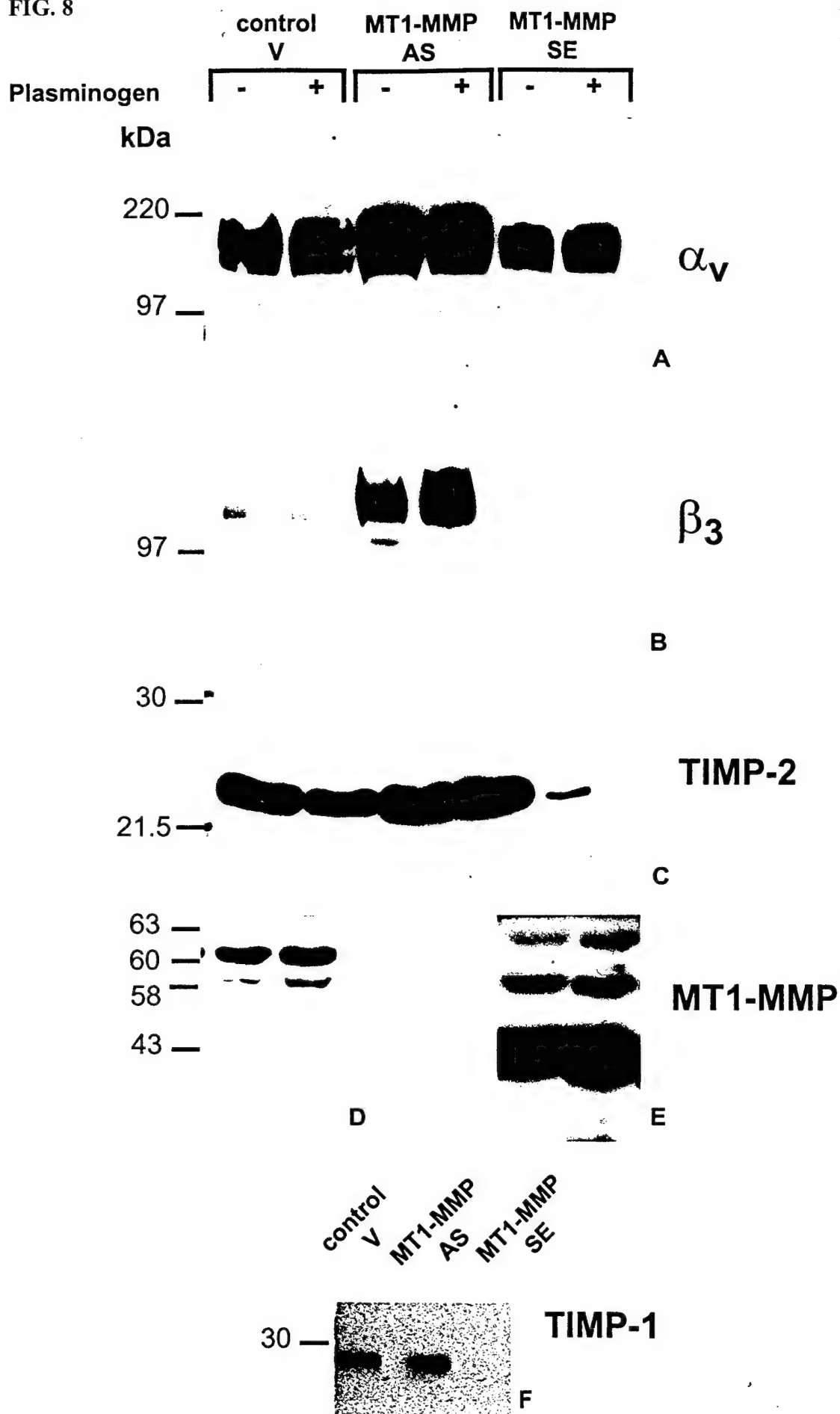




Fig. 9

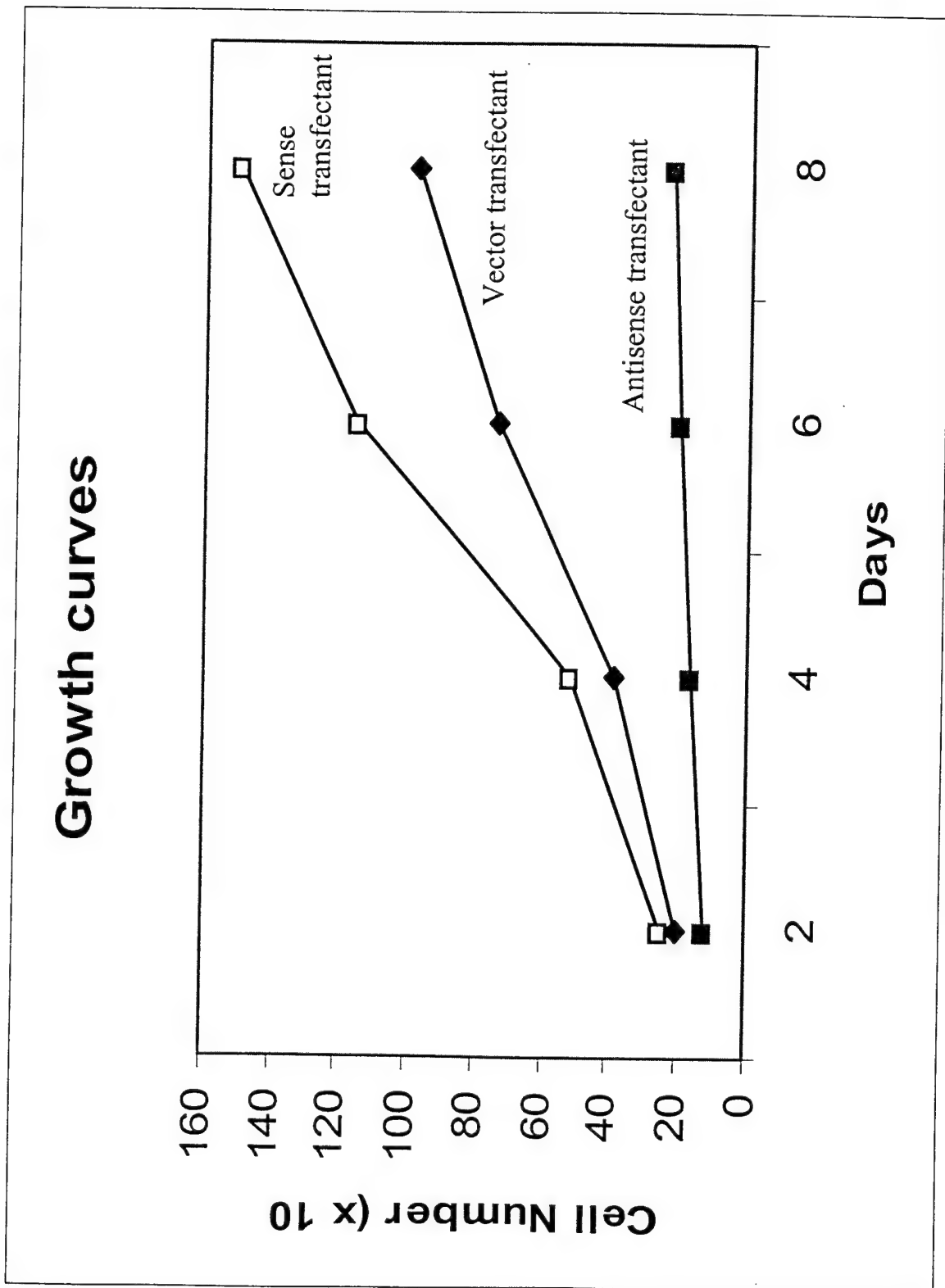
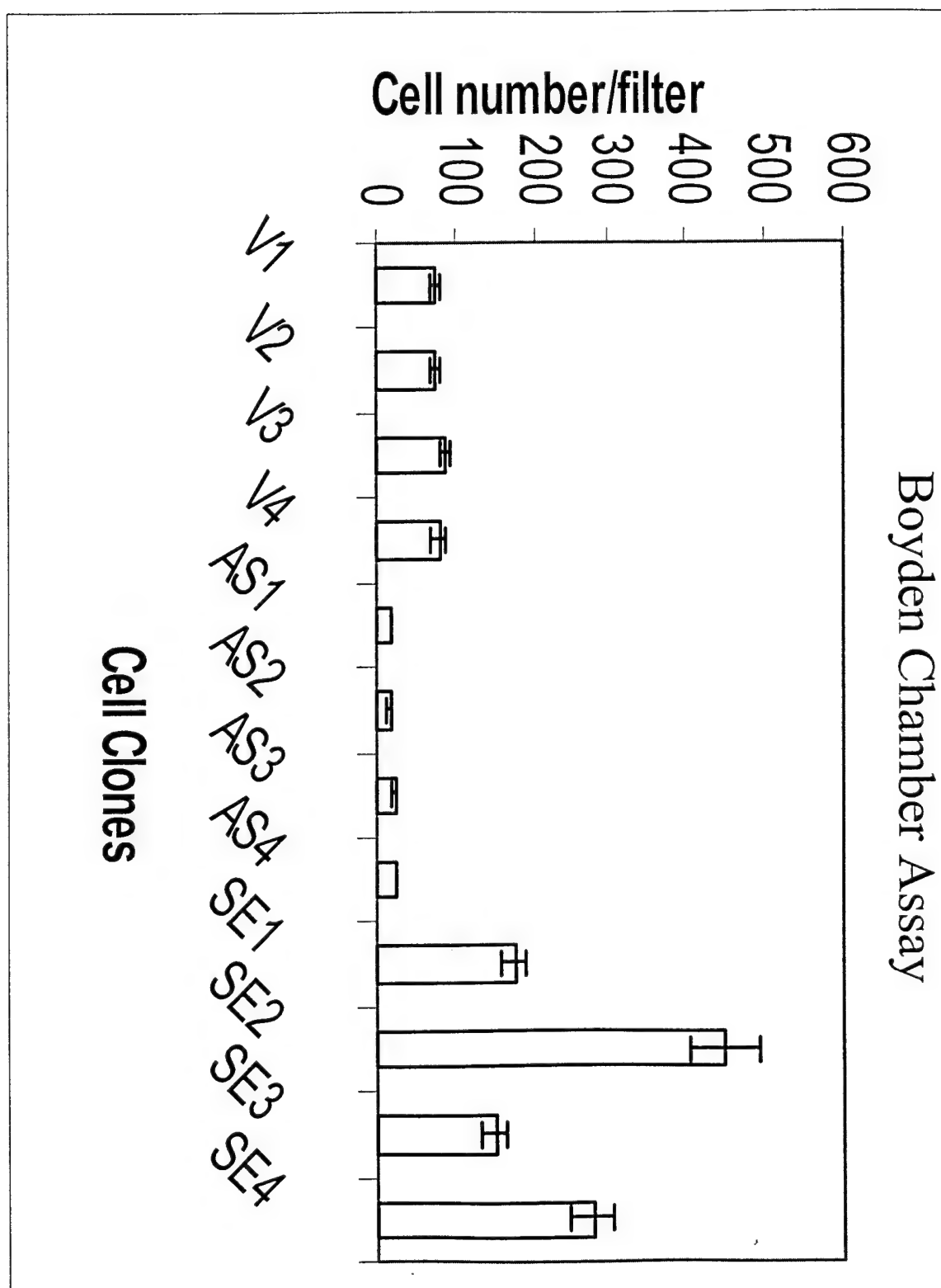


Fig. 10



**MEMBRANE-TYPE 1 MATRIX METALLOPROTEINASE IS REQUIRED FOR  
PLASMIN-MEDIATED ACTIVATION OF GELATINASE A**

Sara Monea\*, Kaisa Lehti<sup>‡</sup>, Jorma Keski-Oja<sup>‡</sup> and Paolo Mignatti\*<sup>†§</sup>

\* Department of Surgery, S.A. Localio Laboratory for General Surgery Research,  
and <sup>†</sup> Department of Cell Biology, NYU School of Medicine, 550 First Avenue,  
New York, New York 10016, USA; <sup>‡</sup> Department of Pathology, The Haartman  
Institute, FIN-00014 University of Helsinki, Finland

**Short title: Progelatinase A activation by plasmin and membrane-type 1 MMP**

<sup>§</sup> To whom correspondence should be addressed:

Paolo Mignatti, New York University School of Medicine, Dept. of Cell Biology, 550  
First Avenue, New York, NY 10016, USA. Telephone: (212) 263 1478; Fax: (212) 263  
0147; e-mail: mignap01@med.nyu.edu

## SYNOPSIS

Membrane-type 1 matrix metalloproteinase (MT1-MMP) has been implicated as a physiological activator of progelatinase A (MMP-2). We previously reported that plasmin treatment of cells results in proMMP-2 activation and increased type IV collagen degradation. Here we analyzed cell surface-associated events involved in plasmin-MT1-MMP interactions in cells transfected with MT1-MMP sense or antisense cDNA. Control, vector-transfected cells expressed significant amounts of MT1-MMP but did not activate proMMP-2. MT1-MMP transfectants expressed high MT1-MMP levels and secreted 68/66 kDa MMP-2 intermediate activation products. Antisense cDNA transfectants had very low levels of MT1-MMP and did not activate proMMP-2. Control cells and MT1-MMP transfectants had much higher levels of cell-associated MMP-2 than antisense cDNA transfectants. Addition of plasmin(ogen) to control or MT1-MMP-transfected cell cultures generated active, 62 kDa MMP-2 but was ineffective with antisense cDNA transfectants. The effect of plasmin(ogen) was blocked by inhibitors of plasmin but not by metalloproteinase inhibitors, implicating plasmin as a mechanism for proMMP-2 activation independent of the activity of MT1-MMP or other MMPs. Plasmin-mediated activation of proMMP-2 did not result from processing of proMT1-MMP and was independent of  $\alpha_v\beta_3$  integrin or TIMP-2 levels. Thus, plasmin can activate proMMP-2 only in the presence of MT1-MMP; however, this process does not require the catalytic activity of MT1-MMP.

## INTRODUCTION

The matrix metalloproteinases (MMPs) are a family of enzymes involved in a variety of physiological and pathological processes that require extracellular matrix remodeling, such as organogenesis, wound repair, tumor invasion and metastasis. Besides few exceptions, all MMPs are secreted in an inactive form, are activated extracellularly by limited proteolytic cleavage and are inhibited by specific tissue inhibitors (TIMPs). ProMMP activation involves the cleavage of the N-terminal "pro" peptide with a subsequent decrease in  $M_r$  of about 10 kD. The net activity of the secreted enzymes is the result of the balance between zymogen activation and interaction of the active forms with TIMPs. The physiological mechanisms that regulate MMP activity in pericellular spaces are not completely understood. MMPs are involved in a cascade of proteolytic reactions that also involve components of the plasminogen activators (PA)-plasmin system. All these enzymes act in concert to degrade most protein components of the extracellular matrix (ECM) [1, 2].

An important role in tumor invasion and metastasis is played by the type IV collagenases/gelatinases (MMP-2 or gelatinase A, and MMP-9 or gelatinase B). MMP-2 and MMP-9 degrade a variety of ECM proteins, including collagen types IV and I, fibronectin, laminin and elastin. In addition, they are potent gelatinases [2-5]. The physiological mechanisms of activation of these enzymes have not been elucidated. MMP-2, in particular, appears to have unique requirements for activation. A variety of data have shown that cell membrane components play a critical role in MMP-2 activation [6-10]. Six membrane-bound proteins - the membrane-type MMPs (MT-MMPs) - have been identified and implicated as physiological activators of proMMP-2. Unlike all the

other MMPs, which are secretory proteins, MT-MMPs are bound to the cell membrane through a C-terminal hydrophobic transmembrane sequence or a glycosylphosphatidylinositol anchor. The transmembrane MT-MMPs therefore have the catalytic domain exposed on the cell surface and a short (20–26 amino acid) cytoplasmic domain. However, soluble MT-MMP forms have also been described in the culture medium of some tumor cell lines [11, 12]. MT-MMPs are expressed in a variety of normal tissues and tumors [13–20]. MT1-, MT2- and MT3-MMP have been implicated as physiological activators of MMP-2 [13, 21]; MT4-MMP is structurally different from the other MT-MMPs and its physiological role is unclear [22]. MT5-MMP, expressed specifically in the brain and during embryonic development, activates progelatinase A when co-expressed in Madin-Darby canine kidney [14]. MT6-MMP is a GPI-anchored protein associated with specific granules of leukocytes and possesses strong gelatinolytic activity [20].

MT1-MMP is expressed by most cell types and tissues. Its activity and substrate specificity have been well characterized [13, 23–26]. MT1-MMP, a 63 kDa protein, processes 72 kDa proMMP-2 into an active form of 64/62 kDa via an intermediate 68/66 kDa form [8, 26–29]. However, this process requires overexpression of MT1-MMP by treatment of cells with concanavalin A, phorbol esters or transforming growth factor beta-1 (TGF $\beta$ -1) [26, 27, 30–32]. In addition, proMMP-2 activation by MT1-MMP requires the presence of TIMP-2, although high concentrations of exogenous TIMP-2 inhibit MMP-2 activation [8, 27]. In the presence of TIMP-2 tri-molecular complexes consisting of MT1-MMP, MMP-2 and TIMP-2 are associated with the membrane of HT1080 fibrosarcoma cells. MMP-2 also binds  $\alpha_v\beta_3$  integrin on the surface of endothelial



cells [33, 34], indicating that MMP-2 interaction with the cell surface can be mediated by multiple binding sites. The C-terminal domain of MMP-2 is required for binding and activation by MT1-MMP [7, 35] as well as for binding to  $\alpha_v\beta_3$ . The relative contribution of  $\alpha_v\beta_3$  and MT1-MMP to proMMP-2 activation has not been elucidated. In addition to activating proMMP-2, recombinant MT1-MMP mutants that lack the transmembrane domain degrade collagens, gelatin, fibronectin and laminin [36].

We have previously reported that components of the urokinase (uPA)-plasmin system are involved in the control of type IV collagenase activity on the cell surface. In cell cultures, physiological concentrations of plasmin activate both proMMP-9 and proMMP-2 without the action of other metallo- or acid proteinases. On the contrary, in soluble phase plasmin degrades both MMP-9 and MMP-2 [10]. To characterize the cell membrane components required for plasmin-mediated activation of MMP-2, we studied potential interactions between MT1-MMP,  $\alpha_v\beta_3$  integrin, TIMP-2 and plasmin. Here we report that proMMP-2 activation by plasmin requires expression but not the catalytic activity of MT1-MMP and is independent of  $\alpha_v\beta_3$  and TIMP-2 levels.

## EXPERIMENTAL

**Materials.** Human plasminogen was purified as described [37]; gelatin-Sepharose was purchased from Pharmacia Biotech AB (Uppsala, Sweden), gelatin from Merck (Darmstadt, Germany), aprotinin, 1,10-phenanthroline, and 4-aminophenylmercuric acetate (APMA) from Sigma (St. Louis, MO), the MMP-substrate, Mca-pro-leu-Gly-Leu-Dpa-Ala-Arg-NH<sub>2</sub>, from Calbiochem-Novabiochem International (Switzerland). Protein concentrations were measured by the Bradford protein assay reagent (Biorad, Melville, NY) using bovine serum albumin (BSA, Sigma) as a standard. Antibody to a 26-residue synthetic peptide corresponding to the C-terminal, intracellular domain of human MT1-MMP (amino acid residues 557-582) has been described. The antibody was purified by affinity chromatography with the antigen coupled to CNBr-activated Sepharose 4B [32]. Antibodies to TIMP-1 and to TIMP-2 were purchased from Calbiochem-Novabiochem International, antibodies to  $\alpha_v$  and to  $\beta_3$  integrin chains from Chemicon (Temecula, CA) and from Transduction Laboratories (Lexington, KY), respectively.

**Cells and culture medium.** Human HT1080 fibrosarcoma cells were originally obtained from the American Type Culture Collection (ATCC, CCL-121) and grown in Dulbecco's minimum essential medium (DME) supplemented with 10% fetal calf serum (FCS), L-glutamine 2 mM, 100 units/ml of penicillin and 100  $\mu$ g/ml of streptomycin (Gibco BRL, Gaithersburg, MD).

**Transfection of HT1080 cells with MT1-MMP cDNA.** The expression plasmids containing nucleotides 1-2369 of MT1-MMP cDNA (pc3SE) or the corresponding antisense cDNA (pc3AS) under transcriptional control by the CMV promoter in the pcDNA3 vector have been described [32]. For stable transfection 8 ml of OPTI-MEM

(Gibco BRL) was gently mixed with 9  $\mu\text{g}$  of either pc3 or pc3SE or pc3AS and 80  $\mu\text{l}$  of Lipofectin (Gibco BRL), and the mixture was incubated at room temperature for 40 min. Subconfluent HT1080 cells in 10-cm dishes were washed twice with serum-free DME. The mixture was added to the cells and incubated at 37° C for 16 h. The cells were washed with DME and incubated with 10 ml of DME supplemented with 10% FCS and 250  $\mu\text{g}/\text{ml}$  of geneticin (Sigma, St. Louis, MO). Geneticin-resistant cell clones were subcultured in DME containing 200  $\mu\text{g}/\text{ml}$  of geneticin. Expression of MT1-MMP by the cell clones was analyzed by Western blotting as described below.

**Preparation of cell extracts and conditioned media.** HT1080 cells were seeded into 10-cm culture dishes at a density of  $2.6 \times 10^7$  cells/dish. The cells were washed twice with phosphate buffer saline (PBS) to remove residual FCS, and incubated for 16 h with 4 ml/dish of serum-free DME with or without 4  $\mu\text{g}/\text{ml}$  of plasminogen and/or the indicated concentrations of proteinase inhibitors. The culture supernatants were centrifuged at 500 x g for 10 min at 22° C. For gelatin zymography the cells were washed twice with PBS, lysed for 10 min on ice with 1 ml /dish of Triton X-100 0.5% (v/v) in Tris-HCl 0.1 M, pH 8.1 (lysis buffer) under constant shaking, and scraped with a rubber policeman. For Western blotting the cells were washed with PBS, scraped with a rubber policeman, centrifuged in an Eppendorf tube for 3 min and resuspended in 100  $\mu\text{l}$  of lysis buffer on ice for 10 min. For the analysis of TIMP-2 or TIMP-1 in cell-conditioned media, 1 ml of serum-free culture supernatant was concentrated with Centricon tubes. The cell lysates were centrifuged at 800 x g for 10 min at 4° C. Conditioned media and cell extracts were immediately analyzed by Western blotting and/or gelatin-zymography.

**Gelatin zymography.** Cell extracts (0.8-1.0 mg of protein in 1 ml) or conditioned media (4 ml) were incubated at 4° C for 1 h in an end-over-end mixer with 25 µl of gelatin-Sepharose equilibrated with 50 mM Tris-HCl, 150 mM NaCl, 5 mM CaCl<sub>2</sub>, 0.02% (v/v) Tween-20, 10 mM EDTA, pH 7.6 [38]. After 4 washes with 1 ml of equilibration buffer containing 200 mM NaCl, the beads were resuspended in 30 µl of 4 X non-reducing Laemmli buffer, and loaded on SDS-8% polyacrylamide gels containing 1 mg/ml of gelatin. After electrophoresis, the gels were washed twice with 200 ml of 2.5% (v/v) Triton X-100 at 22° C for 2 h to remove SDS, and 3 times for 5 min with H<sub>2</sub>O to remove Triton X-100. The gels were incubated in 50 mM Tris-HCl, 0.2 M NaCl, 20 mM CaCl<sub>2</sub>, pH 7.4 at 37° C for 6-12 h, stained overnight with Coomassie Brilliant Blue R-250 0.5% (w/v) in 45% (v/v) methanol, 10% (v/v) acetic acid and destained in the same solution without dye [39]. The M<sub>r</sub>s of the lysis bands were determined by reference to high-molecular mass (14.3 - 200 kDa) standards (Rainbow Markers; Amersham, England).

**Western blotting.** Concentrated conditioned media or cell extracts (80 µg) were electrophoresed in a reducing SDS-polyacrylamide gel and electroblotted to a nitrocellulose membrane (Hybond-C Extra, Amersham). The membrane was prehybridized at 22° C for 1 h or at 4° C overnight in Tris base 20 mM, NaCl 150 mM, 0.1% Tween 20, pH 7.4 (TBS-T) containing 5% milk (Carnation), and hybridized at 22° C for 1 h in TBS-T containing 5% milk and rabbit antibody to either MT1-MMP (1:500) or to the  $\alpha_v$  (1:250) or  $\beta_3$  (1:2,000) integrin chains, or mouse anti-TIMP-1 or anti-TIMP-2 antibody (1:200). The membrane was incubated at 22° C for 45 min in TBS-T containing horseradish peroxidase-labeled anti-rabbit or anti-mouse IgG (Amersham). Each step was followed by extensive washing in TBS-T (4 ml/cm<sup>2</sup>) at 22° C. After removing the TBS-T

buffer, the membrane was incubated for 1 min at 22° C with 0.125 ml/cm<sup>2</sup> of ECL detection solution (Boehringer) and exposed to films (Hyperfilm MP, Amersham) for 10 sec to 5 min.

**MMP activity assay.** The fluorogenic MMP substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH<sub>2</sub>-AcOH was dissolved to 1.0 mM in dimethylsulphoxide as described [40] and stored at 4° C in the dark to avoid photooxidation. To measure gelatinase activity, the substrate was diluted to 10 µM in 50 mM Tris-HCl, 0.2 M NaCl, 20 mM CaCl<sub>2</sub>, pH 7.4 (assay buffer) [39]. Increasing volumes (10 µl to 300 µl) of control medium or cell-conditioned medium were added to 500 µl of assay buffer containing the substrate, and the reaction mixture was incubated at 37° C in the dark for 30 min to 6 h. At the end of the incubation the reaction was blocked by addition of 500 µl of 10% acetic acid. The reaction product was measured as described, using a FluoroMax-2 fluorimeter (excitation max 325 nm, emission max 393 nm) [39]. APMA (1 mM)-treated cell-conditioned medium or conditioned medium supplemented with EDTA (10 mM) was used as positive or negative control, respectively. As a control for the specificity of the assay for the gelatinases, conditioned medium was pretreated with gelatin-Sepharose as described above and the supernatant of the resin, depleted of the gelatinases, was tested in the assay. Samples and controls were assayed in duplicate. The total amount of gelatinase activity present in the conditioned media was assessed by pretreating samples with APMA as described above. By gelatin zymography APMA treatment resulted in complete activation of MMP-2 and MMP-9 [10; and data not shown).

## RESULTS

### **MT1-MMP expression and proMMP-2 activation in HT1080 cells transfected with MT1-MMP sense or antisense cDNA**

Non-transfected HT1080 cells and clones of HT1080 cells transfected with MT1-MMP cDNA, its antisense cDNA or the vector alone were characterized by Western blotting with antibody to the intracellular domain of MT1-MMP. Consistent with previous reports [32, 41-43], multiple forms of MT1-MMP with  $M_r$ s 63,000, 60,000, 58,000 and 43,000 were detected (Fig. 1). The  $M_r$ s of the 63,000 and 60,000 bands are consistent with those of pro- and active MT1-MMP, respectively [32, 41, 42]. The 58 kDa immunoreactive protein represents an activation product of MT1-MMP; the 43 kDa band, whose actual  $M_r$  is 38,000 based on amino acid sequencing [42], represents a degradation product of MT1-MMP [32, 42-44].

Non-transfected cells (not shown) or cells transfected with the vector alone constitutively expressed 60 kDa and 58 kDa MT1-MMP (Fig. 1 A). Under comparable immunoblotting conditions the levels of these MT1-MMP forms were dramatically reduced in all the clones of cells transfected with the antisense cDNA, relative to those of control, vector-transfected cells (Fig. 1 C). All the clones of cells transfected with the sense cDNA expressed 60 kDa and 58 kDa MT1-MMP, in addition to the 63 kDa proenzyme and the 43 kDa band (Fig 1 E), consistent with previous findings that the generation of this peptide is associated with overproduction of MT1-MMP [32, 42, 43].

The transfected cells were also characterized for gelatinase expression and activation by gelatin-zymography of conditioned medium or cell extracts. MMP-9 was present in the conditioned medium of all the transfected cell clones primarily in its inactive, 92 kDa form. In contrast, the pattern of MMP-2 forms showed significant



differences among the different transfectants. Non-transfected HT1080 cells (not shown) and cells transfected with the vector alone, which constitutively express active (60 kDa) MT1-MMP, secreted MMP-2 only in its inactive, 72 kDa form as did antisense cDNA transfectants that express very low amounts of MT1-MMP (Fig. 1 B and D). In contrast, in the conditioned medium of cells transfected with MT1-MMP cDNA 68/66 kDa intermediate activation forms of MMP-2 could be detected in addition to the 72 kDa proenzyme (Fig. 1 F). Consistent with previous reports, the generation of the active forms of MMP-2 correlated with the overexpression of 63 kDa and 60 kDa MT1-MMP and with production of the 43 kDa form of MT1-MMP [42, 43]. Thus, the constitutive expression of active (60 kDa) MT1-MMP was not sufficient to activate proMMP-2, a process that requires high levels of MT1-MMP expression.

Gelatin zymography of cell extracts (Fig. 2) showed MMP-2 associated with cells transfected with MT1-MMP cDNA or with the vector alone. Very low levels of MMP-2 were associated with extracts of antisense MT1-MMP cDNA transfectants that express virtually no MT1-MMP, although these cells secreted amounts of 72 kDa MMP-2 comparable to those of the other cell clones (Fig. 2 B). This finding indicated a role for MT1-MMP as a major MMP-2 binding site on the cell membrane.

**TIMP-2 and  $\alpha_v\beta_3$  integrin expression in sense or antisense MT1-MMP cDNA transfectants**

Because TIMP-2 and  $\alpha_v\beta_3$  integrin have been implicated in the cell surface binding and activation of proMMP-2 [27, 33, 34], a panel of MT1-MMP transfectant cell clones were also characterized for the expression of these proteins (Fig. 3). By Western blotting control cells and antisense MT1-MMP cDNA transfectants secreted comparable amounts of TIMP-2 (Fig. 3A, lanes 1-4). In contrast, the levels of TIMP-2 in the conditioned medium of MT1-MMP-transfected cells were dramatically lower (Fig. 3A, lanes 5-7). Cell extracts of all the clones contained comparable amounts of TIMP-2 (Fig. 3 A), showing that the low TIMP-2 levels in the conditioned medium of MT1-MMP transfectants did not result from increased TIMP-2 binding to MT1-MMP or the ECM. Similar differences were observed in the amounts of secreted TIMP-1, which was undetectable in the conditioned medium of MT1-MMP transfectants (see below, Fig 7 F). Likewise, the levels of the  $\alpha_v$  and  $\beta_3$  integrin chains were considerably lower in the MT1-MMP transfectants than in the other cell clones (Fig. 3A). Thus, no correlation was apparent between TIMP-2 or  $\alpha_v\beta_3$  levels and the cell surface binding and activation of proMMP-2.

To investigate whether the downregulation of  $\alpha_v\beta_3$  and TIMP-2 resulted from increased MMP activity in the MT1-MMP transfectants, the cells were grown in the presence of the MMP inhibitor Marimastat (10  $\mu$ M). As shown in Fig. 3 B, in the presence of Marimastat the conditioned medium of all the clones contained comparable levels of TIMP-2, implicating MMP activity in TIMP-2 downregulation. In contrast, Marimastat had no effect on  $\alpha_v\beta_3$  levels, indicating that  $\alpha_v$  and  $\beta_3$  gene expression may be downregulated in our MT1-MMP transfectants.

### **Plasmin-mediated activation of proMMP-2 in cells expressing differing levels of MT1-MMP**

Plasmin has been shown to activate proMMP-2 in the presence but not in the absence of cells, suggesting a role for the cell surface in this process [10]. To investigate the potential role of MT1-MMP in plasmin-mediated activation of proMMP-2, the transfected cell clones were incubated in the presence or absence of plasminogen (4  $\mu\text{g/ml}$ ) for 16 h. HT1080 cells secrete high levels of uPA that rapidly convert plasminogen into plasmin [10; and data not shown). Gelatinase activation was analyzed both by degradation of a specific fluorogenic substrate and by gelatin zymography, as described under Experimental.

To assess the linearity of the fluorogenic assay, increasing volumes (10 – 300  $\mu\text{l}$ ) of medium conditioned by non-transfected HT1080 cells in the absence or in the presence of plasminogen (4  $\mu\text{g/ml}$ ) were diluted in 500  $\mu\text{l}$  of assay buffer and incubated in the presence of the substrate (10  $\mu\text{M}$ ) for 30 min to 3 h. The assay was linear with volumes of conditioned medium ranging 10-100  $\mu\text{l}$  and incubation times ranging 0.5 – 3.0 h. (Fig. 4). Addition of EDTA to the reaction mixture or depletion of the gelatinases from the conditioned medium by gelatin-Sepharose chromatography as described under Experimental blocked substrate degradation (Fig. 5), showing the specificity of the assay for the gelatinases. Consistent with previous findings [10], medium conditioned by non-transfected HT1080 cells in the presence of plasmin(ogen) showed a 4.5-fold increase in gelatinase (MMP-2 and MMP-9) activity relative to medium conditioned in the absence of plasminogen (Fig. 4).

With vector-transfected cells the activity measured in the presence of plasmin(ogen) (~40% of the total, APMA-activatable gelatinase activity) was similar to that of MT1-MMP cDNA-transfected cells in the absence of plasmin(ogen) (Fig. 5). The levels of total, APMA-activatable gelatinase activity in these two clones were comparable (see legend to Fig. 5). With MT1-MMP transfectants that overexpress MT1-MMP addition of plasmin(ogen) increased the gelatinase activity to approximately 75% of the total APMA-activatable activity. In contrast, with antisense cDNA transfectants that express very low levels of MT1-MMP plasmin only increased gelatinase activity to approximately 20% of the total activity (Fig. 5). The total APMA-activatable gelatinase activity of antisense cDNA transfectants was approximately 30% higher than in the other two clones, showing that the lower activity measured in the presence of plasmin(ogen) was not because of higher TIMP-2 or TIMP-1 levels. With all the three clones, the activity was completely abolished by EDTA or by pretreatment of the conditioned media with gelatin-Sepharose, showing that the activity measured by this assay in medium conditioned by the different transfectants was mediated exclusively by gelatinases.

By gelatin zymography medium conditioned in the absence of plasmin(ogen) by cells overexpressing MT1-MMP (Fig. 6, MT1-MMP SE) showed three MMP-2 bands of 72, 68/66 and 64/62 kDa. Control, vector-transfected cells (V) and antisense cDNA transfectants (MT1-MMP AS) showed only one 72 kDa band. Addition of plasmin(ogen) to the culture medium of antisense cDNA transfectants had no effect on proMMP-2 activation, although it generated active 84/82 kDa forms of MMP-9 (Fig. 6 C). In contrast, addition of plasmin(ogen) to the culture medium of control cells or MT1-MMP transfectants generated active, 64/62 kDa MMP-2 forms (Fig. 6 A, B and D). With

control, vector-transfected cells that constitutively express MT1-MMP, the plasmin-mediated conversion of 72 kDa to 64/62 kDa MMP-2 was less efficient than with the MT1-MMP transfectants (Fig. 6 D). With the MT1-MMP overexpressing cells plasmin fully converted the 68/66 kDa intermediate activation product of MMP-2 to active, 64/62 kDa MMP-2 (Fig. 6 D). Thus, plasmin generates active MMP-2 in cells that express MT1-MMP but has no effect on proMMP-2 secreted by cells with very low levels (or virtually devoid) of MT1-MMP.

The observation that in the presence of plasmin(ogen) the antisense cDNA transfectants had no active MMP-2 but secreted 84/82 kDa forms of MMP-9 (Fig. 6 C) showed that the low level of gelatinase activity measured by the fluorogenic substrate assay in medium conditioned by these cells in the presence of plasmin(ogen) was mediated only by MMP-9. MT1-MMP transfectants had a level of MMP-9 much lower than those of the other two clones. In the presence of plasmin(ogen) MT1-MMP transfectants did not show activation of MMP-9 (Fig. 6), an unexpected but reproducible effect. Thus, the activity measured by the fluorogenic assay in the conditioned medium of these cells was mediated only or predominantly by MMP-2.

These findings suggested that the effect of plasmin on MMP-2 activation could be mediated either indirectly by cleavage/activation of cell surface proteins such as MT1-MMP,  $\alpha_v\beta_3$  or TIMP-2, or directly by activation of cell surface-associated proMMP-2. To test this hypothesis, we characterized MT1-MMP,  $\alpha_v\beta_3$  and TIMP-2 by Western blotting of extracts of cells grown in the presence or absence of plasminogen. With control HT1080 cells that express 60 kDa and 58 kDa MT1-MMP (Fig. 7 D), and with MT1-MMP transfectants that also express the 63 kDa proenzyme (Fig. 7 E) plasmin had

no effect on the generation of active (60 kDa and 58 kDa) MT1-MMP or on the formation of the 43 kDa form of MT1-MMP that correlated with proMMP-2 activation. Similar results were obtained by surface labeling wild-type HT1080 cells or MT1-MMP transfectants by the biotin-avidin method [42] followed by immunoprecipitation after different incubation times (30 min to 6 h) in the presence or absence of varying amounts of plasmin(ogen) (data not shown). Plasmin also had no effect on the levels of  $\alpha_v$  or  $\beta_3$  integrin chains expressed by the three cell lines (Fig. 7 A, B) but dramatically downregulated the amount of TIMP-2 in the conditioned medium of the MT1-MMP transfectants, which secrete a low amount of this inhibitor (see Fig. 3 A). However, plasmin did not appear to significantly reduce the levels of TIMP-2 in the other two clones, which expressed considerably higher amounts of the inhibitor (Fig. 7 C). By immunoblotting, degradation products of TIMP-2 could not be detected in the medium of any of the cell clones. Thus, plasmin activation of proMMP-2 does not appear to be mediated by proMT1-MMP activation or by cleavage or degradation of  $\alpha_v\beta_3$  integrin or TIMP-2.

To characterize the relative contribution of plasmin and MT1-MMP to proMMP-2 activation the transfected cell clones were grown for 16 h in the presence or absence of plasmin(ogen) and different proteinase inhibitors, and MMP-2 activation was analyzed by gelatin zymography of cell-conditioned medium. Addition to MT1-MMP transfectants of 1,10-phenanthroline (10  $\mu$ g/ml) or Marimastat (data not shown), which inhibit metalloproteinases, resulted in decreased 68/66 kDa MMP-2 (Fig. 6 B). This observation is consistent with previous findings that the processing of proMMP-2 to 68/66 kDa is MT1-MMP-dependent and that conversion to fully active, 62 kDa MMP-2 occurs

through autocatalysis [29]. Addition of plasminogen in the presence or absence of the metalloproteinase inhibitors resulted in the conversion of the 68/66 kDa form to 64/62 kDa MMP-2, indicating that plasmin can catalyze the generation of active 62 kDa MMP-2 under conditions in which MT1-MMP activity and/or autocatalysis are inhibited. Conversely, the effect of plasmin(ogen) was inhibited by addition of aprotinin (100  $\mu\text{g/ml}$ ), a serine proteinase inhibitor that blocks plasmin activity (Fig. 6 A and B). As addition of aprotinin alone has no effect on the gelatinases [10; and data not shown], these findings showed that plasmin directly catalyzes the generation of 64/62 kDa MMP-2 from the 68/66 kDa form generated by MT1-MMP and from the 72 kDa proenzyme.

## DISCUSSION

The data reported show several features of MT1-MMP-plasmin interactions in proMMP-2 activation: a) MT1-MMP and plasmin act in concert to activate proMMP-2; b) plasmin activation of proMMP-2 requires the expression but not the catalytic activity of MT1-MMP; c) cell-associated proMMP-2 is activated by plasmin, whereas secreted proMMP-2 is not; d) proMMP-2 activation by plasmin does not result from plasmin-mediated MT1-MMP activation and is independent of  $\alpha_v\beta_3$  integrin or TIMP-2 levels. These conclusions are based on the following observations.

HT1080 cells constitutively express MT1-MMP. Transfection with MT1-MMP sense or antisense cDNA generated clones of cells with differing levels of MT1-MMP. For the sake of simplicity we will refer to the amounts of MT1-MMP constitutively expressed by non-transfected or vector-transfected cells as "basal" levels, as opposed to the "high" levels present in cells transfected with the sense cDNA or to the "low" levels expressed by the antisense cDNA transfectants.

Clones of HT1080 cells that expressed high levels of MT1-MMP constitutively activated MMP-2. Consistent with previous reports, proMMP-2 activation correlated with the presence in these cells of 63 kDa MT1-MMP and/or with the generation of a 43 kDa (38 kDa based on aa sequence) degradation or activation product [32, 42]. However, non-transfected HT1080 cells or cells transfected with an empty vector expressed significant levels of MT1-MMP but no active MMP-2. Addition of plasmin(ogen) to these cells resulted in proMMP-2 activation and in a several fold increase in gelatinase activity. In contrast, with cells transfected with antisense cDNA, which expressed very low levels (or



were virtually devoid) of MT1-MMP, activation of proMMP-2 by plasmin did not occur. These findings show that high levels of MT1-MMP are necessary for proMMP-2 activation in the absence of plasmin. With basal levels of MT1-MMP (insufficient to activate proMMP-2) the gelatinase can be activated by plasmin; however, proMMP-2 activation by plasmin does not occur in the presence of low levels (or virtually in the absence) of MT1-MMP. Thus, basal levels of MT1-MMP are necessary and sufficient for proMMP-2 activation by plasmin.

$\alpha_v\beta_3$  integrin and TIMP-2 have been implicated in MMP-2 binding to and activation on the cell surface [27, 33, 34]. Our MT1-MMP transfectant cell clones expressed different levels of  $\alpha_v\beta_3$  integrin, which did not correlate with proMMP-2 activation in the presence or in the absence of plasmin(ogen). Cells that expressed high levels of MT1-MMP but no  $\alpha_v\beta_3$  integrin (MT1-MMP transfectants) had cell-associated MMP-2 and activated this gelatinase. Plasmin enhanced proMMP-2 activation in these cells. Plasmin also induced MMP-2 activation in control, vector-transfected cells that had relatively high levels of  $\alpha_v\beta_3$  integrin; however, it had no such effect with antisense cDNA transfectants that expressed high levels of this integrin but very low levels of MT1-MMP. With our transfected HT1080 cell clones MMP-2 was associated with extracts of cells that expressed no detectable  $\alpha_v\beta_3$  integrin but had high levels of MT1-MMP (MT1-MMP transfectants). In contrast, cells that expressed high amounts of the integrin but very low levels of MT1-MMP (antisense cDNA transfectants) had very low levels of cell-associated MMP-2. These findings indicate that, although MMP-2 can interact with multiple binding sites on the cell surface, MT1-MMP appears to play a major role in localizing the gelatinase on the cell membrane.

Likewise, with our transfected HT1080 cells TIMP-2 levels did not appear to be relevant for plasmin-mediated activation of proMMP-2. Gelatinase activation occurred in cells that express high levels of MT1-MMP and very low TIMP-2 levels (MT1-MMP transfectants) but not in cells expressing higher levels of the inhibitor and very low levels of MT1-MMP (antisense cDNA transfectants). However, plasmin activated proMMP-2 in cells that expressed TIMP-2 levels comparable to that of antisense cDNA transfectants but higher levels of MT1-MMP. Low TIMP-2 levels may account for the high MMP-2 activity of the MT1-MMP transfectants (Fig. 5). However, proMMP-2 activation was also detected by zymography (Fig. 6), showing that MMP-2 processing indeed occurs in the presence of plasmin. In addition, increased gelatinase activity was also obtained by addition of plasmin(ogen) to control cells that have relatively high TIMP-2 levels (Fig 4 and 5).

TIMP-2 downregulation in MT1-MMP transfectants was abolished by addition of the MMP inhibitor Marimastat, whereas  $\alpha_v\beta_3$  integrin downregulation was not. Because this phenomenon was consistent in several transfectant clones, it is unlikely to simply reflect clonal variability and suggests that  $\alpha_v\beta_3$  downregulation may result from MT1-MMP overexpression through non-catalytic mechanisms mediated by the transmembrane and/or the cytoplasmic domain of this MMP.

It is noteworthy that TIMP-2 and TIMP-1 can also be degraded by plasmin. This finding indicates a novel role for plasmin in the regulation of MMP activity. Plasmin not only can activate some MMPs, including MMP-1, MMP-3 and MMP-9 [45-52], but it can also increase their activity by downregulating MMP inhibitors. In the case of MMP-

2, whose activation depends on TIMP-2 levels [27], plasmin control of TIMP-2 results in the regulation of both MMP-2 activation and catalytic activity.

HT1080 cells that expressed MT1-MMP had cell-associated MMP-2; cells transfected with the antisense cDNA, which expressed very low levels of MT1-MMP, had much lower amounts of cell-associated MMP-2. These cells expressed high levels of  $\alpha_v\beta_3$  integrin. Previous results have shown that cells treated with ionomycin, which blocks proMT1-MMP activation, have no surface-associated MMP-2 and do not activate the gelatinase even in the presence of PMA [42]. These findings are consistent with the reported role of MT1-MMP as a binding site for proMMP-2•TIMP-2 complex on the cell membrane [27]. In our experiments with cells that had very little cell-associated proMMP-2 the gelatinase could not be activated by plasmin, showing that plasmin-mediated activation requires binding of proMMP-2 to the cell membrane through its interaction with MT1-MMP.

With our HT1080 cell transfectants plasmin-mediated activation of proMMP-2 occurred in the presence of the metalloproteinase inhibitors 1, 10-phenanthroline (Fig. 6) or Batimastat (data not shown). This observation confirms our previous report that plasmin activation of proMMP-2 does not require the action of metallo- or acid proteinases [10]. The MMP inhibitors we used blocked the generation of 68/66 kDa MMP-2 by cells that overexpressed MT1-MMP, showing that they did indeed block MT1-MMP activity. The cleavage of proMMP-2 by MT1-MMP into a 68/66 kDa intermediate activation product has been proposed to trigger autocatalytic activation of the gelatinase to the fully active 64/62 kDa form [26-29]. However, plasmin generated 64/62 kDa MMP-2 in the presence of 1,10 phenanthroline or Batimastat, which inhibit

MT1-MMP activity and MMP autocatalysis. Thus, whereas expression of MT1-MMP is necessary for cell binding and activation of proMMP-2, its catalytic activity and autocatalysis do not appear to be involved in plasmin-mediated activation of proMMP-2.

Our results also show that plasmin has no effect on MT1-MMP activation, as demonstrated by Western blotting analysis and by surface labeling and immunoprecipitation (data not shown). Others have reported that plasmin catalyzes the conversion of proMT1-MMP into a catalytically active enzyme able to activate proMMP-2 [52]. These results were obtained with a recombinant GST-proMT1-MMP fusion protein in soluble phase; it is possible that native, membrane anchored MT1-MMP is protected from proteolytic cleavage by plasmin and is activated by other intracellular or extracellular mechanism(s) [36]. Because plasmin had no effect on the MT1-MMP expressed by our HT1080 cells, its effect on proMMP-2 activation results from a direct or indirect action on the gelatinase. ProMMP-2 can be activated by stromelysin-1 (MMP-3) [53] or by matrilysin (MMP-7) [54]. Our HT1080 cells express no MMP-3, as assessed by casein zymography and Western blotting with specific antibody (data not shown). It is possible that plasmin indirectly activates proMMP-2 by "unmasking" MT1-MMP on the cell membrane, e.g. by cleaving cell surface molecules associated with it and making it more available for interaction with the substrate. However, our finding that plasmin activates proMMP-2 in the presence of 1, 10 phenanthroline, EDTA [10] or Batimastat strongly indicates that plasmin acts directly on proMMP-2 or proMMP-2•TIMP-2 complex. A possible mechanism of action could also consist of plasmin cleavage of the C-terminal, hemopexin-like domain of proMMP-2. Such cleavage would not affect the N-terminal pro domain, but would account for the decrease in  $M_r$  observed by

zymography. In addition, cleavage in the hemopexin domain would prevent TIMP-2 binding, which would result in increased activity of MMP-2 activated by other mechanisms. However, in such a case active MMP-2 should have  $M_r$  lower than 62,000 as activation would entail cleavage of both the pro domain and of the C-terminal peptide. We cannot rule out the hypothesis that TIMP-2 degradation by plasmin may contribute to increasing MMP-2 activation and activity. However, addition of plasmin(ogen) to antisense cDNA-transfected cells, which have TIMP-2 levels comparable to that of control cells, did not result in proMMP-2 activation and increased gelatinase activity. This finding rules out the hypothesis that TIMP-2 degradation is the only mechanism by which plasmin mediates increased MMP-2 activation and activity.

Addition of plasminogen to control cells or antisense cDNA transfectants resulted in proMMP-9 activation but had no effect on the proMMP-9 secreted by MT1-MMP transfectants (Fig. 6). This effect was observed several times in a reproducible manner. The reason for the lack of proMMP-9 activation by plasmin in MT1-MMP transfectants is not clear. We have previously reported that MMP-9 activation also requires the presence of the cell surface [10]. We hypothesize that the high levels of MT1-MMP in transfected cells localize higher amounts of MMP-2 on the cell membrane than in non-transfected cells. MMP-2 localized on the cell membrane may compete with MMP-9 for plasmin either because it is in excess of MMP-9 or because MT1-MMP-bound MMP-2 is activated by plasmin more efficiently than MMP-9 (e.g. MMP-2 may be spatially closer than MMP-9 to cell surface-associated plasmin).

MT1-MMP has been proposed to be a "physiological activator" of proMMP-2. However, proMMP-2 activation by MT1-MMP occurs only in cells that overexpress

MT1-MMP following transfection or treatment with PMA or Con A, reagents that also upregulate other proteinases in many cell types [8, 26, 31, 32]. In addition, although MT1-MMP is expressed by a variety of cell types, only some of these cells express active MMP-2 [55-57]. These findings raise important questions as to the quantitative and qualitative requirements for MT1-MMP-mediated activation of proMMP-2: is upregulation of MT1-MMP required for proMMP-2 activation or are other factors involved? In a variety of tumors MT1-MMP expression has been correlated with the presence of active MMP-2 [17, 18]. However, all these studies have not quantitated MT1-MMP relative, for example, to normal tissues; in addition, they have not considered the possible concomitant presence of other factors implicated in MMP-2 activation, including plasminogen activators and/or other MMPs. Whereas increased expression of MT1-MMP may represent one mechanism for MMP-2 activation, our findings show that under conditions in which MT1-MMP is not upregulated plasmin can activate proMMP-2 by acting in concert with MT1-MMP. As the catalytic activity of MT1-MMP does not appear to be required for plasmin-mediated activation of proMMP-2, our results indicate that a primary role for MT1-MMP may be to serve as a membrane binding site for MMP-2•TIMP-2 complex. A consistent body of experimental evidence has shown the role of the cell surface in the regulation of the proteolytic cascade involved in tissue remodeling [1, 58]. Both MMP-2 and MMP-9 are located to the cell surface [8, 10, 59]. Binding of uPA to its cell membrane receptor (uPAR) strongly accelerates proMMP-2 and pro-MMP-9 activation; plasmin(ogen) binding to the cell surface is also required for gelatinase activation [10]. Membrane vesicles shed by HT1080 cells possess surface-bound uPA and gelatinases; vesicle-associated gelatinases are also activated by plasmin

[60]. Thus, components of the plasminogen activator (PA)-plasmin system represent an alternative mechanism for the cell surface activation of proMMP-2 under conditions in which MT1-MMP is not upregulated.

The cell surface binding of all components of the PA-plasmin-MMP cascade has two major implications: the juxtaposition of all the reactants accelerates molecular interactions; in addition, it may protect molecular species from uncontrolled proteolytic degradation. Plasminogen activators are expressed by many cell types, including those that produce MMP-2 and MT1-MMP. In addition, in tissues virtually all components of the proteolytic cascade can be produced by different cell types. Relatively high concentrations of plasminogen are present in all tissues, including the basal layers of epidermis [61-62]. The production of small amounts of plasminogen activators affords the generation of high local concentrations of plasmin. Thus, *in vivo* the expression of low levels of MT1-MMP insufficient to activate proMMP-2 directly may afford gelatinase activation by providing a cell membrane binding site that permits limited cleavage of proMMP-2 by plasmin and/or possibly other proteinases.

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**FOOTNOTE**

The abbreviations used in this paper are: DME: Dulbecco's minimum essential medium; ECM: extracellular matrix; EDTA: ethylene diamino tetraacetic acid; FCS: fetal calf serum; MMP: matrix metalloproteinase; MT-MMP: membrane-type metalloproteinase; PA: plasminogen activator; PBS: phosphate buffered saline; SDS: sodium dodecyl sulfate; TGF $\beta$ -1: transforming growth factor beta-1; TIMP: tissue inhibitor of metalloproteinases; uPA: urokinase plasminogen activator.

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## FIGURE LEGENDS

**Fig. 1. Characterization of MT1-MMP and gelatinases in clones of HT1080 cells transfected with MT1-MMP sense or antisense cDNA or with the vector alone.** Western blots (A, C, E) of Triton X-100 cell extracts (80  $\mu$ g) and gelatin zymograms (B, D, F) of serum-free medium conditioned by clones of cells transfected with the empty vector (V), or with antisense (AS) or sense (SE) MT1-MMP cDNA. Western blotting with anti-MT1-MMP antibody and gelatin zymography were performed as described under Experimental. The films shown in A, C and E were all exposed for 1 min and developed under the same conditions. Molecular weight markers are shown in kDa on the left of panels A, B, E and F. These experiments were repeated twice with comparable results.

**Fig. 2. Characterization of cell-associated gelatinases in clones of HT1080 cells transfected with MT1-MMP sense or antisense cDNA or with the vector alone.** Zymographic analysis of the gelatinases associated with Triton X-100 cell extracts (A) or serum-free conditioned media (B) of HT1080 cell clones transfected with MT1-MMP cDNA (SE), the corresponding antisense cDNA (AS), or the empty vector (V). MT1-MMP expression by the indicated cell clones is shown in Fig. 1. Conditioned media and cell extracts were analyzed by gelatin zymography as described under Experimental. Molecular masses are shown in kDa on the left of each panel. This experiment was repeated twice with comparable results.

**Fig. 3. Characterization of MT1-MMP, gelatinases,  $\alpha_v\beta_3$  integrin and TIMP-2 in clones of HT1080 cells transfected with either vector alone (control V) or with MT1-MMP antisense (AS) or sense cDNA (SE).** Confluent cells were grown for 16 h in serum-free medium in the absence (panel **A**) or in the presence (panel **B**) of Marimastat 10  $\mu$ M. The cells were lysed with Triton X-100 0.5% (v/v) in Tris-HCl 0.1 M, pH 8.1. Eighty  $\mu$ g of Triton X-100 cell extracts was analyzed by Western blotting with antibodies to MT1-MMP, to the  $\alpha_v$  or  $\beta_3$  integrin chains, or to TIMP-2. Concentrated conditioned medium was analyzed by gelatin zymography and Western blotting with antibody to TIMP-2. Molecular masses are shown in kDa on the left of each panel. These experiments were repeated three times with comparable results.

**Fig. 4. Effect of plasminogen on the gelatinase activity of HT1080 cells.** Medium conditioned by non-transfected HT1080 cells in the presence (●) or in the absence of 4  $\mu$ g/ml of plasminogen (○) was assayed for gelatinase activity with the fluorogenic assay described under Experimental. The activity of the conditioned medium was blocked by EDTA or by pretreatment with gelatin-Sepharose, showing that the assay is specific for the gelatinases (see Fig. 5). **A.** Time course. One hundred  $\mu$ l of cell-conditioned medium was assayed for the indicated time. **B.** Dose-dependence. The indicated volumes of cell-conditioned medium were incubated with the substrate for 3 h. These experiments were repeated twice with comparable results.



**Fig. 5. Effect of plasmin(ogen) and MT1-MMP on gelatinase activity.** Gelatinase activity of serum-free medium conditioned by control, vector-transfected cells, or by MT1-MMP sense or antisense cDNA-transfected cells. The cells were grown for 16 h in serum-free medium in the absence or in the presence (+) of 4  $\mu\text{g/ml}$  of plasminogen (Plg). MMP activity was measured as described under Experimental. Control, non-conditioned medium (DME) was tested in the presence or absence of plasminogen (4  $\mu\text{g/ml}$ ) and uPA (50 mU/ml) as a negative control. Medium conditioned in the presence or absence of plasminogen was assayed in the absence or in the presence of either APMA (1 mM) or EDTA (50 mM), or after treatment with gelatin-Sepharose (gel-Seph) as described under Experimental. The activity measured in the presence of APMA was considered as the total gelatinase activity (100%) of the conditioned medium. The actual fluorometric readings of the APMA-treated samples were: control HT1080, 1765; antisense MT1-MMP, 3093; sense MT1-MMP, 2135. The activity was abolished by EDTA or by pretreatment of conditioned medium with gelatin-Sepharose, showing that the assay is specific for the gelatinases. Mean and experimental variability of duplicate samples are shown. This experiment was repeated three times with comparable results.

**Fig. 6. Effect of plasmin(ogen) and MT1-MMP on proMMP-2 activation.** Gelatin zymography of serum-free medium conditioned by control, vector-transfected cells (**A**), MT1-MMP overexpressing cells (**B**) or antisense MT1-MMP cDNA-transfected cells (**C**). The cells were grown for 16 h in serum-free medium in the absence (-) or in the presence (+) of 4  $\mu\text{g/ml}$  of plasminogen, with or without addition of 100  $\mu\text{g/ml}$  of aprotinin and/or 10  $\mu\text{g/ml}$  of 1, 10-phenanthroline. **D.** The samples shown in panels A and B were run in the same gelatin zymogram to compare the MMP-2 activation products. The samples were analyzed by gelatin zymography as described under Experimental. Molecular masses are shown in kDa on the left of each panel. These experiments were repeated five times with comparable results.

**Fig. 7. MT1-MMP,  $\alpha_v\beta_3$  integrin, TIMP-2 and TIMP-1 expression in HT1080 cell transfectants grown in the absence or in the presence of plasmin(ogen).** HT1080 cells transfected with either the vector alone (V), or with MT1-MMP sense (SE) or antisense MT1-MMP cDNA (AS) were incubated for 16 h in serum-free medium with (+) or without (-) plasminogen (4  $\mu\text{g/ml}$ ). Eighty  $\mu\text{g}$  of Triton X-100 cell extracts was analyzed by Western blotting with antibodies to the  $\alpha_v$  (A),  $\beta_3$  (B) integrin chains or to MT1-MMP (D and E) as described under Experimental. Conditioned medium (1 ml) was concentrated and analyzed by Western Blotting with antibodies to TIMP-2 (C) or to TIMP-1 (F, medium conditioned in the absence of plasminogen). Molecular masses are shown in kDa on the left of each panel. These experiments were repeated three times with comparable results.

FIG. 1

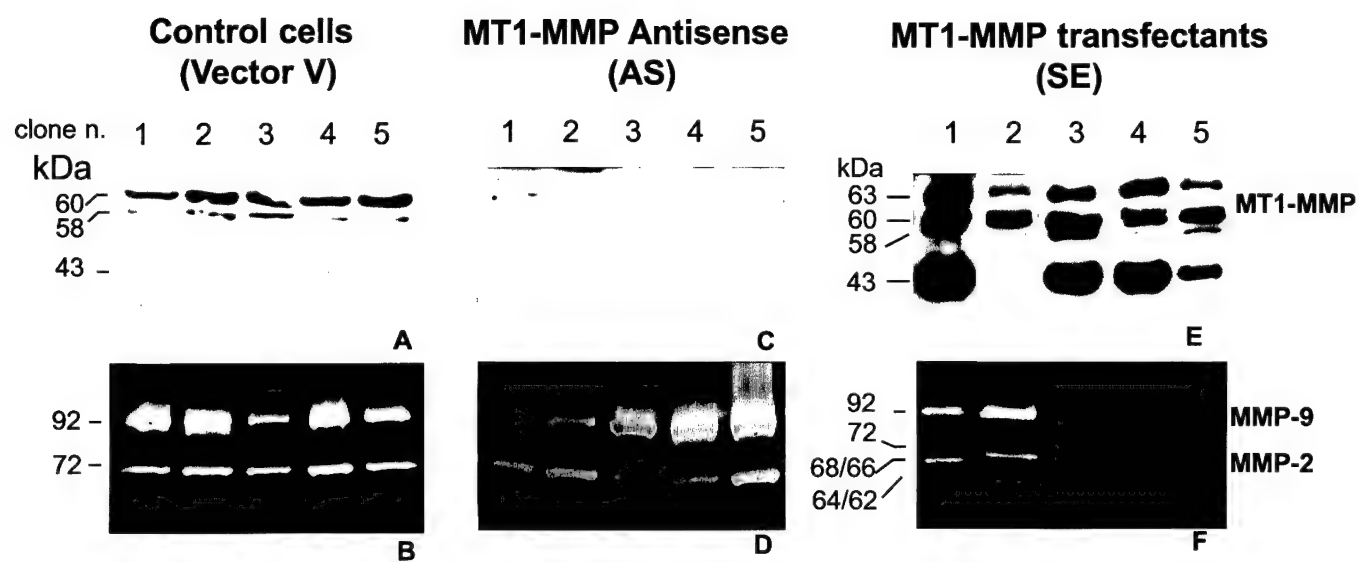


FIG. 2

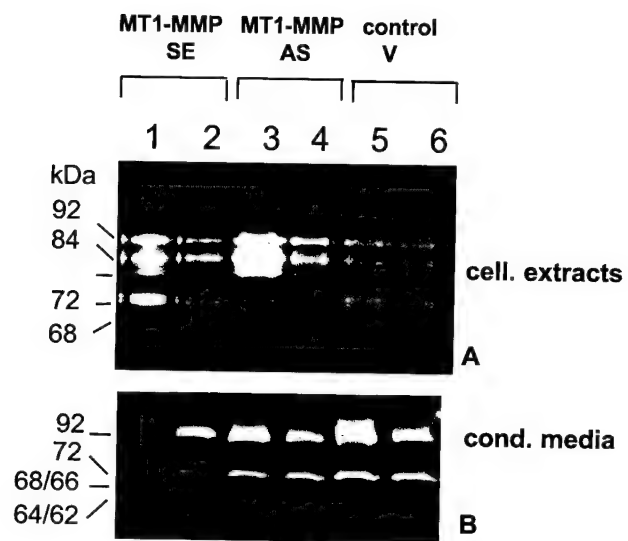
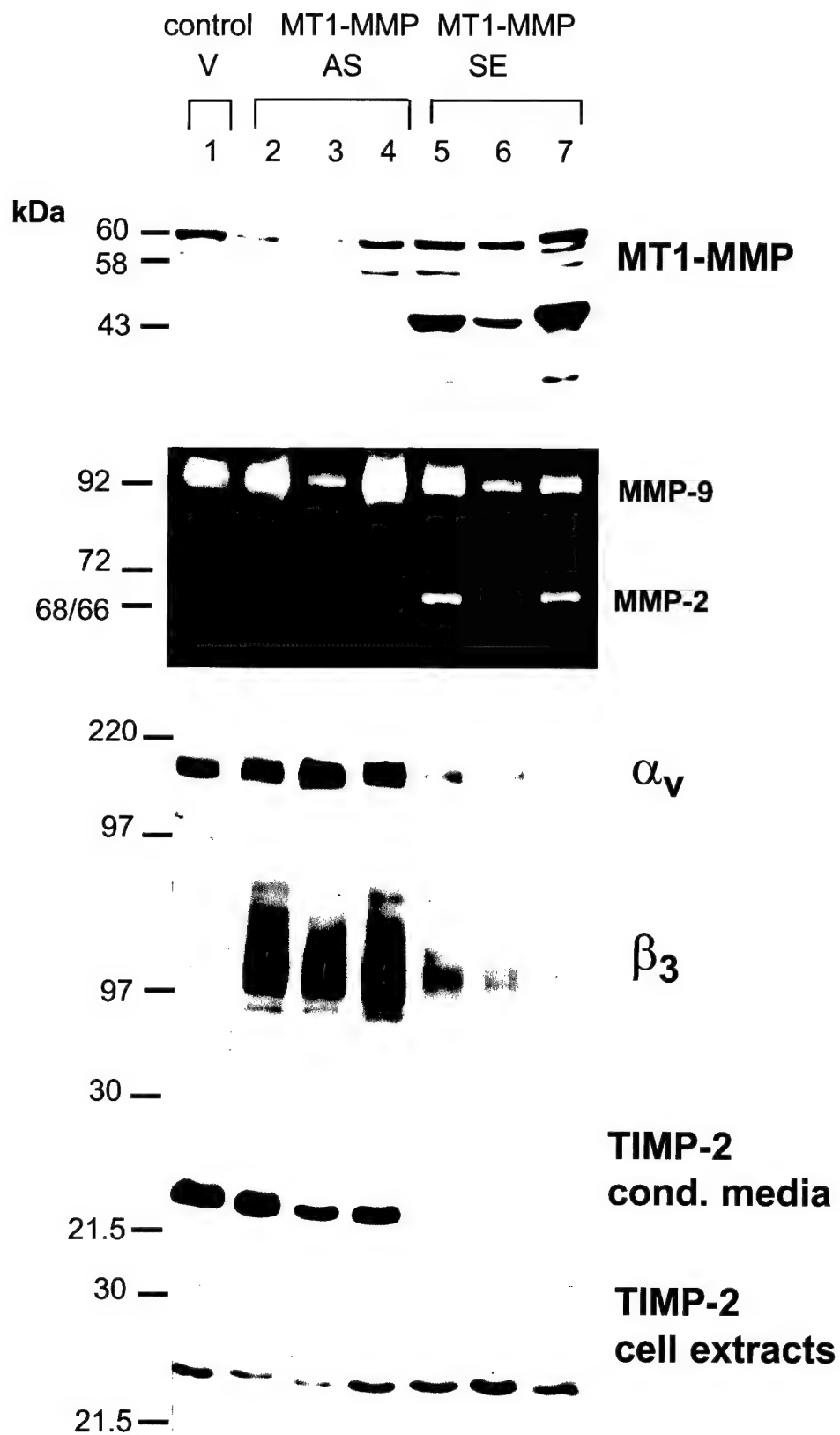


FIG. 3 A



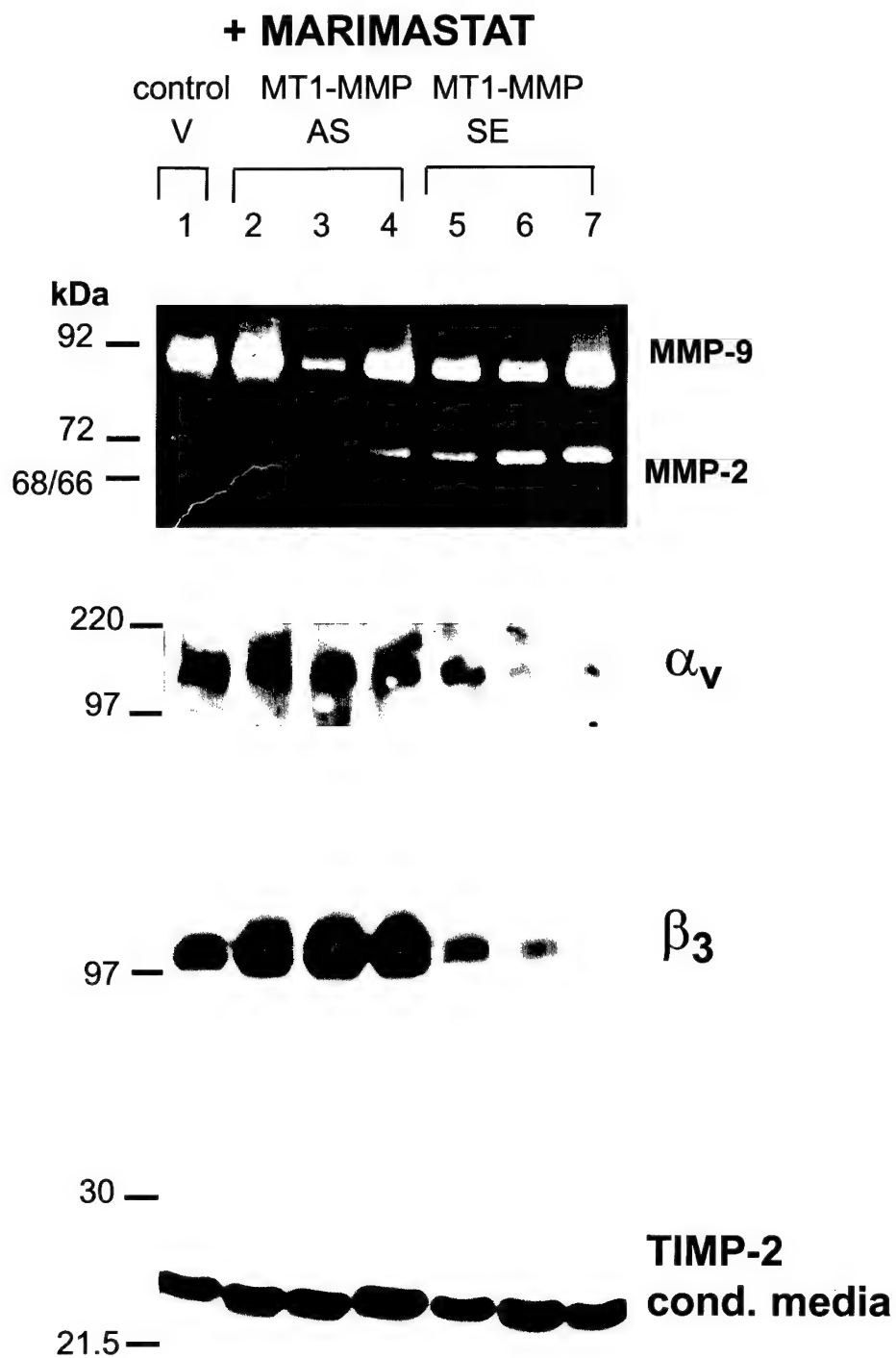


FIG. 3 B

FIG. 4

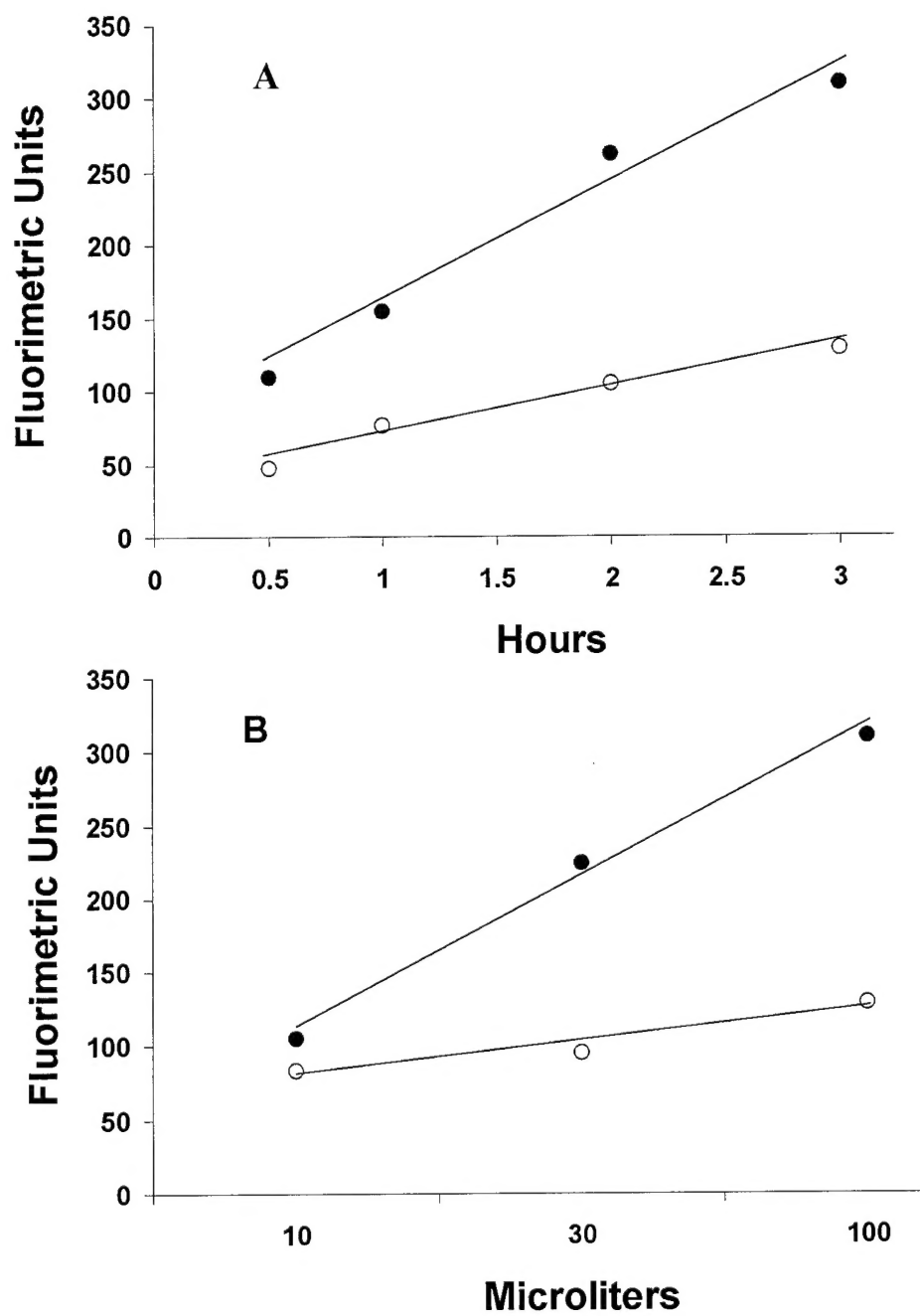


FIG. 5

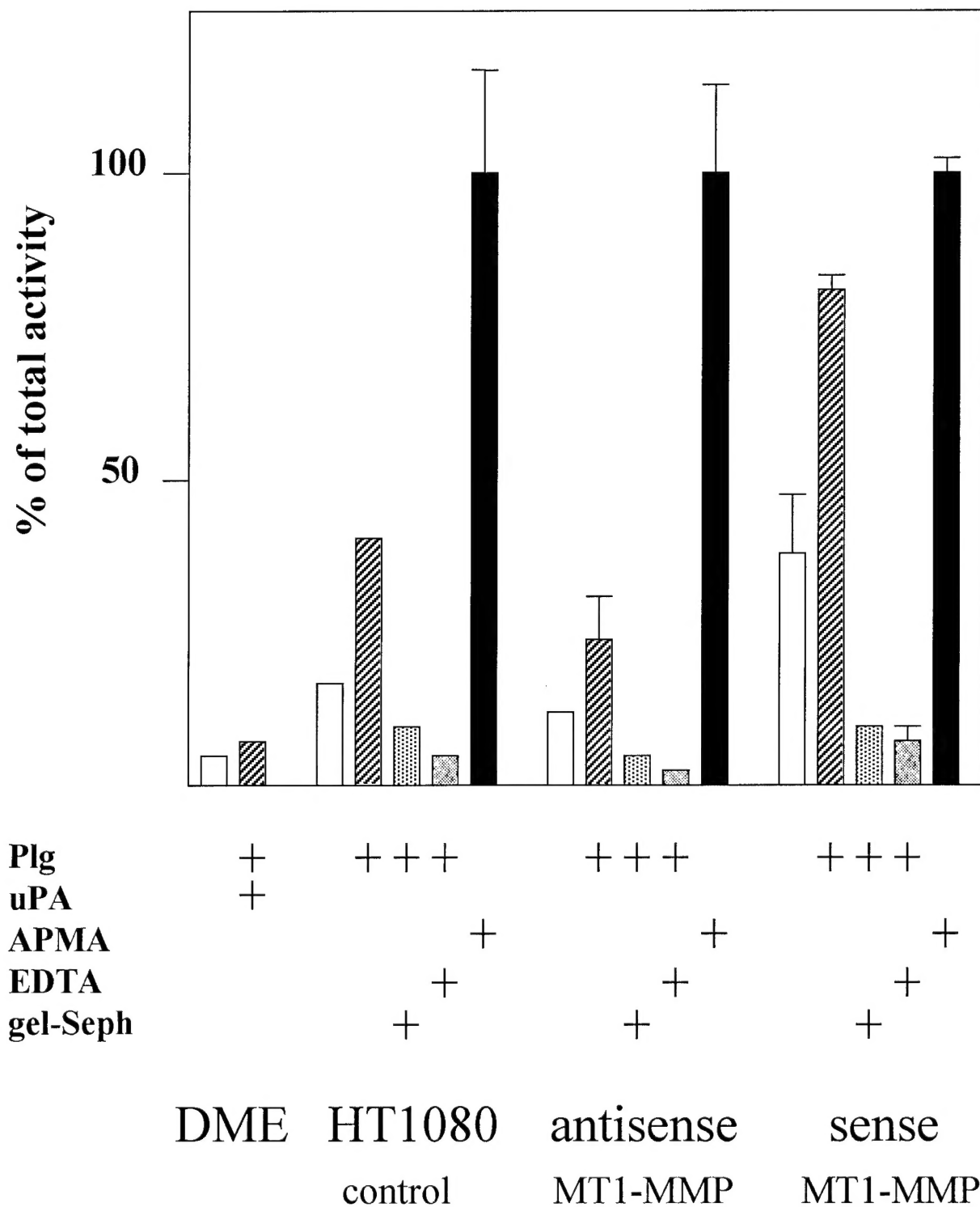
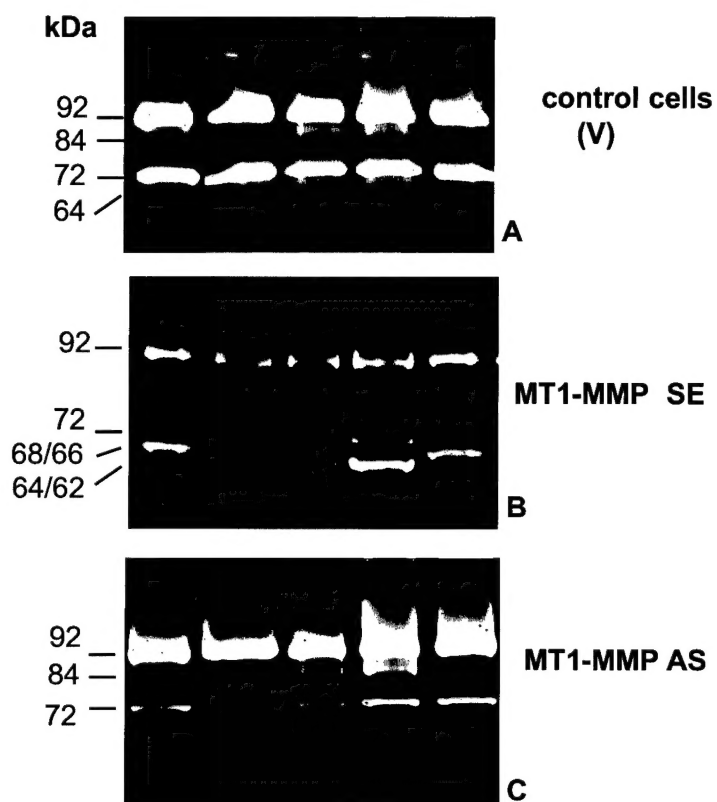


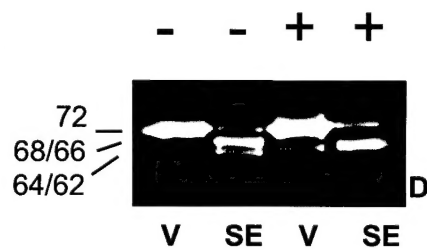


FIG. 6

Phenanthroline	-	+	+	-	-
Plasminogen	-	-	+	+	+
Aprotinin	-	-	-	-	+



Plasminogen



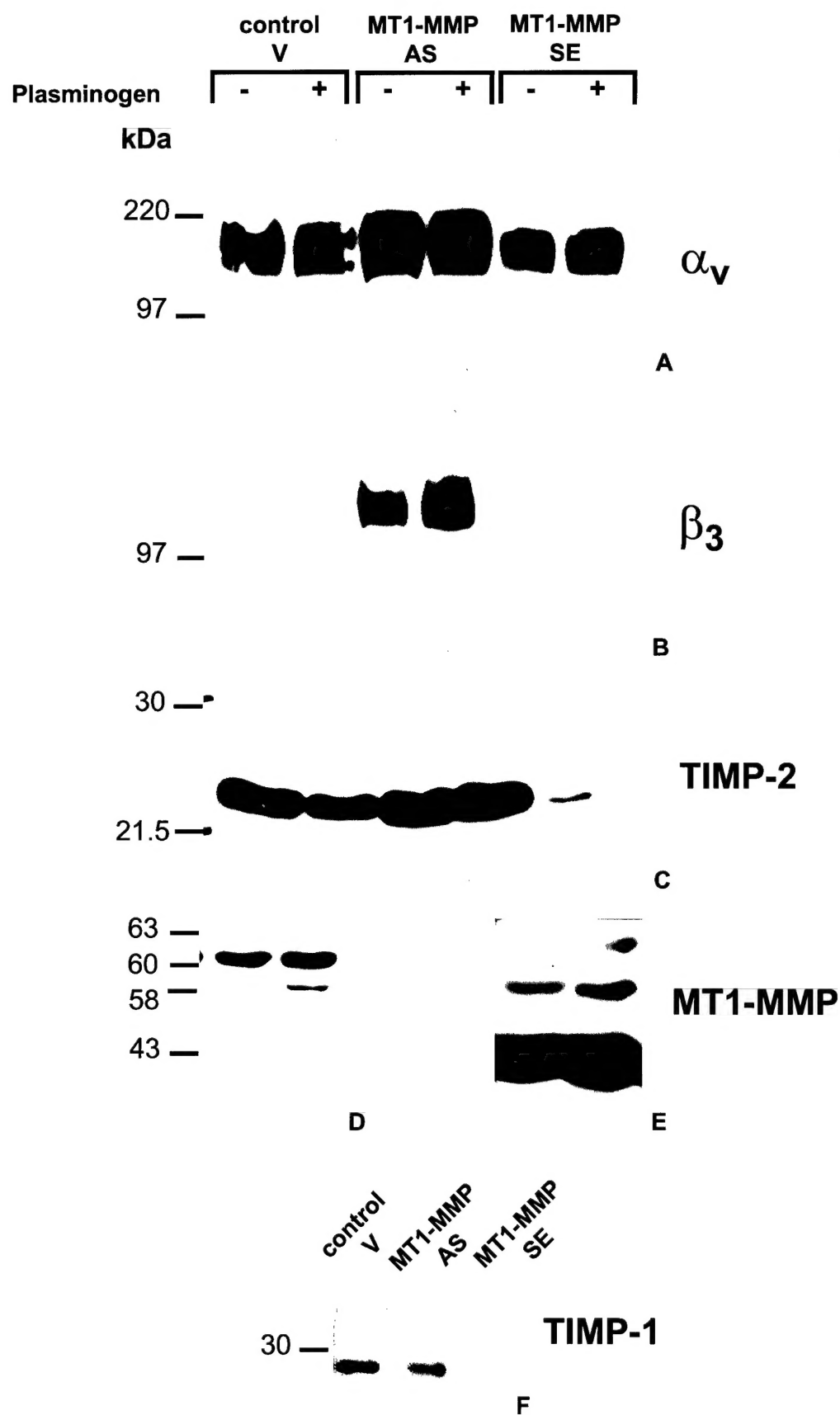


FIG. 7